Plasticizers as endocrine disruptors: the case of the endocannabinoid system in teleost species.

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Plasticizers as endocrine disruptors: the case of the Endocannabinoid system in teleost species.

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A la meua benvolguda familia,

muns pares i man germana

"Caminante no hay camino, se hace camino al andar"

(M. Machado)

"The best way to predict the future, is to create it"

(A. Lincoln)

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ABSTRACT

Plasticizers, as Bisphenol A (BPA) and Di-isononyl phthalate (DiNP), are chemicals added to the plastics to improve their performance, which recently, turned into a ubiquitous in the environment due to the high rate of use and manufacture of plastic, and hence, of the plasticizers. BPA and DiNP have been found in all environmental matrices, being bioavailable for organisms and reported as endocrine-disrupting chemicals (EDCs). On the other hand, the endocannabinoid system (ECS), a novel lipid signaling system lately defined, has been proposed as a new target for the potential effects of the EDCs. The ECS has been described in several species and it has an essential role for the well-being of the organisms. For that, the main goal of the present project was to assess the effects of BPA and DiNP in the ECS of *Danio rerio* and *Sparus aurata*. To accomplish this objective *D. rerio* and *S. aurata* were chronically treated (3 weeks) with BPA and DiNP, separately, via water or via food respectively. Generally, the results for both species, showed a deregulation of the ECS at central (brain) and peripheral level (liver and gonads) at mRNA and protein level, the alteration of the hepatic lipid metabolism and the biochemical composition of liver in both species. In the gonads, the reproductive performance measured as fertility rate, the gonadal morphology and sexual hormone levels were also altered by the treatments of both pollutants. In conclusion, the chronic exposure to environmental concentrations of BPA and DiNP induced alterations at the ECS pathway in *Danio rerio* and *Sparus aurata*. Finally, the ECS can be considered as new target for EDCs such as BPA and DiNP.

INTRODUCTION

a) THE ENDOCANNABINOID SYSTEM (ECS)

The beginning of the study of the Endocannabinoid system is settled in 1964, when Gaoni and Mechoulam (Gaoni and Mechoulam, 1964) reported the main active component of cannabis, the THC (tethrahydrocannabinol), named as "cannabinoid", acting on the brain. Since the activity of exogenous cannabinoids was revealed, all the implicated network was named as the "endocannabinoid system" (ECS). Moreover, THC was described as a lipid working as a neurotransmitter, with a rare structure for such type of molecules, mainly water – soluble immerged in an aqueous solution (Alger, 2013). Since then, the hypothesis of a new binding sites different from the "typical" receptors in brain emerged after the effects of dose-dependent response to cannabinoids were documented on depression and analgesia. Such hypothesis was confirmed in 1990 when Matsuda and coworkers identified an orphan G protein-coupled receptor in the brain which was bound by cannabinoids, lately named as endocannabinoid receptor type I (CB1) (Matsuda et al., 1990). Few years later, in 1993, a second receptor was discovered, the endocannabinoid receptor type II, namely endocannabinoid receptor 2 (CB2) (Munro et al., 1993). However, the existence of such specific receptors for cannabinoids let the researchers hypothesize about the existence of specific ligands.

In this line, the first ligand identified was the N-arachidonoylethanolamine or Anandamide (AEA), Arachidonic acid with ethanolamine (Devane et al., 1992), and thereafter, the same working group described a second ligand, the 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995). Both molecules were described to act as neurotransmitters, but possessing different characteristics from the classical neurotransmitters. Indeed, AEA and 2-AG are not "pre-synaptically" synthetized and released, but post-synaptically, working as a retrograde synaptic signal and synthetized on demand following some physiological stimuli. Their release was described as Ca^{2+} dependent, since their *de novo* formation is induced

by membrane depolarization. After their biosynthesis in the cytosol, AEA and 2-AG are released into the extracellular space where they act mostly through CB1 and CB2. Once the activity has been detected, the endocannabinoids from the extracellular space, are reuptake very rapidly through the plasma membrane and degraded by specific enzymes in the cytosol.

CB1 is a seven pass-transmembrane receptor coupled with a G-protein with a well-defined structure, considered one of the most abundant in the mammalian brain (Piomelli, 2003), and also expressed in a large number of peripheral tissues (*i.e*. fat, gastrointestinal tract, cardiovascular system, liver, pancreas, bones, skin, muscle, reproductive system and immune system among others) controlling a plethora of physiological processes. AEA acts as a partial or full agonist of CB1 (Pacher et al., 2006), while 2-AG is a CB1 full agonist (Hillard, 2000). Activation of CB1, usually pre-synaptically, modulates the release of other neurotransmitters, such as the dopamine, the noradrenaline or the gama-aminobutyric acid (GABA) (Schlicker and Kathmann, 2001).

CB2 is also a seven pass-transmembrane G-protein-coupled receptor, sharing with CB1 the 44% overall identity. CB2 is largely expressed at peripheric level, mainly in the immune cells, although it has been reported in liver, gastrointestinal track, bones or reproductive system, among others. In addition, CB2 is detected in the central nervous system but at lower levels respect CB1 (Van Sickle et al., 2005). AEA is also a ligand of CB2 although with a lesser efficacy, while 2-AG acts as full agonist.

Another G-protein-coupled receptor has been recently suggested as a novel endocannabinoid receptor, the G protein-coupled receptor 55 (GPR55), which is activated by AEA, Palmitoylethanolamide (PEA) and 2-AG (Ryberg et al., 2007).

AEA and 2-AG also exert a promiscuous behavior since they also bind non-cannabinoid receptors as the transient receptor potential vanilloid type I ion channel (TRPV1) activated by AEA (Högestätt et al., 1999) and oleoylethanolamide

(OEA) (Nagy et al., 2014), but no by 2-AG (Högestätt et al., 1999). In addition, the peroxisome proliferator-activated receptors (PPAR) can be activated by OEA (Fu et al., 2003), PEA (Lo Verme et al., 2004), AEA, AEA metabolites and 2-AG (Sun and Bennett, 2007).

As reported by De Petrocellis and colleagues (De Petrocellis et al., 2004), any molecule acting as an endogenous mediator requires enzymes for its biosynthesis and degradation. In particular, AEA is biosynthesized from membrane phospholipids precursors, specifically, from the phosphatidylethanolamine (PE) by two sequential enzyme reactions, catalyzed by an N-acyltransferase and N-acyl-phosphatidyethanolamine phospholipase D (NAPE-PLD). AEA formation rather than being exclusively of NAPE-PLD activity, can be also obtained through the a/b-hydrolase domain containing 4 (ABDH4) (Simon and Cravatt, 2006) and Phospholipase A2 (PLA2) (Sun et al., 2004) enzymatic activity.

The enzyme fatty acid amide hydrolase (FAAH) is in charge of the hydrolysis of principally AEA, but also 2-AG, to arachidonic acid and ethanolamine or glycerol, respectively, being the major catabolic route for anandamide degradation at least in mammalian tissues (Sugiura et al., 2002). FAAH is located on the intracellular membrane and can hydrolyze other fatty amides as OEA and PEA. Another mechanism for the inactivation of AEA is the enzymatic oxygenation, being AEA the substrate of Prostaglandin-endoperoxide synthase 2/cyclooxygenase-2 (PTGS2/COX-2) and resulting in the eventual production of other bioactive molecules such as prostaglandins-ethanolamides (Yu et al., 1997).

Regarding 2-AG, it is at the cross road of multiple pathways of lipid metabolism (Piomelli, 2003) as well as it is the unique monoacylglycerol having arachidonic acid at the 2nd position of the glycerol backbone (Sugiura et al., 2002).

The precursors of 2-AG are also phospholipids such as the phosphatidic acid (PA) or phosphoinositides (PI) which are catalyzed by DAG lipase (DGLA) through the hydrolysis of the intermediate, 2-arachidonate – containing diacylglycerol (DAG). However, as for AEA, another alternative pathway has been described for the production of 2-AG by the phospholipase A1 (PLA1) (Piomelli, 2003). Regarding the degradation of 2-AG, in addition to FAAH, the monoacylglycerol lipase (MGLL) is one of the main enzymes in charge of the degradation of 2-AG, resulting in arachidonic acid and glycerol. MGLL plays an essential role in the rapid elimination of this endocannabinoid due to the variety of activities exerted by 2-AG (Sugiura et al., 2002). In addition, 2-AG can be inactivated by additional pathways, such by the a/b-hydrolase domain 6 and 12 (ABHD6; ABHD12) (Blankman et al., 2007); by anabolic enzymes as kinases or by enzymatic oxygenation (PTGS2/COX-2) (Kozak et al., 2000) which results in the formation of prostaglandin-glycerol esters.

In addition to AEA and 2-AG, an important role is also exerted by the "endocannabinoid-like mediators" or "AEA-related *N*-acylethanolamines" as PEA and OEA, commented above. Both are known also to perform an "entourage" role due to PEA and OEA can "enhance the action of endogenous anandamide through an increase of the affinity for the receptors or decreasing the degradation of anandamide" (Ho et al., 2008; Lam et al., 2010). Indeed, PEA and OEA synthesis share the same pathway than AEA being also an alternative substrate for FAAH (Jonsson et al., 2001; Maurelli et al., 1995).

In this context, OEA has been identified as a peripheral satiety and lipolytic factor via the peroxisome proliferator-activated receptor alpha (PPARa) (Fu et al., 2003; Guzmán et al., 2004). Instead, PEA has been described as anti-inflammatory and anti-nociceptive substance *in vivo* also via PPAR (Lo Verme et al., 2004).

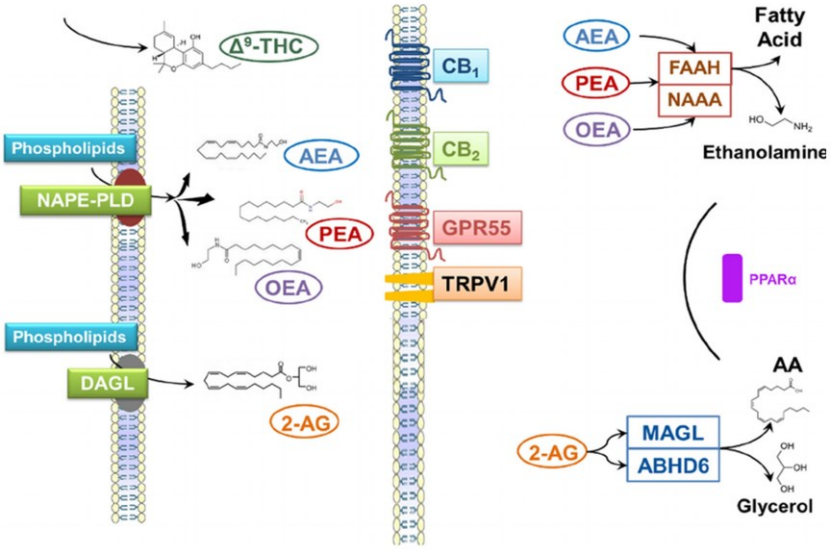

Figure 1. Schematic overview of the ECS (Adapted from Cani et al., 2014).

As we aforementioned, the presence of ECS is not only restricted to the brain, but it possesses a broad distribution throughout the body, produced on demand and acting nearby of the site of biosynthesis (Piomelli, 2003).

ECS is involved in a plethora of physiological and pathological roles, being the correct tone of ECS essential for the well-being of the organisms. ECS tone is mostly the result of the regulation of parallel enzymatic cascades which are ubiquitously expressed. Since the cannabinoid receptors and endocannabinoids are involved in a plethora of physiological activities and are present in a large diversity of tissues, this research project will focus on the hepatic, gonadal and central ECS.

At central level (**brain**), the endocannabinoids work at synaptic level as neuromodulators (Cota, 2007) and among their different functions, the endocannabinoids are deeply implied in the regulation of food intake. Interestingly, Di Marzo and Matias (Di Marzo and Matias, 2005) suggested that ECS controls food intake at two levels: reinforcing the motivation to find and consume food by

reward mechanisms and inducing appetite by regulating levels of orexigenic and anorexigenic mediators

Controversially, it was though that only a central regulation existed for the appetite control, however, such idea changed over the years assuming a cross-talk among the brain and the peripheral tissues (*i.e*. liver) in the control of energy balance. Anyhow, deregulation of the ECS elements implies an imbalance for the organism. For instance, pharmacological blockade of CB1 receptors strongly reduced food consumption in rats (Colombo et al., 1998) however an increase of AEA or 2-AG rises food intake via CB1 (Williams and Kirkham, 1999; Williams and Kirkham, 2002). In fact, a sustained hyperactivity of the ECS let to contribute to the development of obesity and metabolic syndromes (Di Marzo and Matias, 2005).

The ECS in the **liver** is usually lower expressed than in other tissues, however, under pathological conditions, its expression can increase playing a critical role in liver diseases (Maccarrone et al., 2015). Indeed, the hepatic ECS might contribute to fatty liver through disruption of the hepatic lipogenic and lipolytic pathways and insulin signaling (Osei-Hyiaman et al., 2008). In addition, the hepatic endocannabinoid tone may be influenced by several stimuli leading to an ECS deregulation as the starting signal of the hepatic disease. In fact, CB1 activation in hepatocytes promotes lipogenesis *in vivo* and *in vitro* activating the sterol regulation element binding transcription factor 1 (SREBF1), a key transcription factor for lipogenesis, and the fatty acid synthase (FASn). In contrast, mice deficient in CB1 are resistant to high-fat diet obesity (Osei-Hyiaman et al., 2005) as well as hepatocytes treated with CB1 antagonist. CB1 agonists not exclusively increase the gene expression of fatty acids synthesis, but inhibits fatty acid oxidation as well (Jourdan et al., 2012). Moreover, steatosis has been linked to insulin resistance, indeed, the activation of CB1 in the hepatocytes suppress the metabolism of insulin (Liu et al., 2012) while CB1 knockout or pharmacological CB1 blockade are

associated with an improvement of the insulin sensitivity (Osei-Hyiaman et al., 2008).

Regarding CB2, the second-discovered endocannabinoid receptor, it has been implicated in liver fibrosis, fatty liver disease and acute ischemic liver injury (Mallat et al., 2011). In obese mice, CB2 agonist enhanced hepatic steatosis (Deveaux et al., 2009; Louvet et al., 2011), moreover CB2 has been described to exert an anti-inflammatory role possessing the CB2 agonist a therapeutic potential in fatty liver diseases (Maccarrone et al., 2015).

In the **gonads**, the ECS has been mainly studied in mammals, existing few references of the gonadal ECS in non-mammalians organisms including teleost (Cottone et al., 2013; Ruggeri et al., 2007; Valenti et al., 2005). In any case, at least in mammals, a correct tone of endocannabinoids is essential for several aspects regarding female reproduction, such as oviductal embryo transport, placentation and parturition, being mainly regulated by CB1 (Maccarrone et al., 2015; Meccariello et al., 2014). Regarding the male, ECS is essential for germ cell differentiation, Sertoli cells and Leydig cells activities and sperm motility (Grimaldi et al., 2013).

It is well stablished that the reproductive events and the ECS are linked, accordingly, a cross-talk among sex steroids and endocannabinoids has been recently demonstrated. Indeed, estrogens can induce alterations in the action of endocannabinoids on hypothalamic – pituitary axis (Meccariello et al., 2014; Scorticati et al., 2004). Specifically in mouse Sertoli cells of testes, in the FAAH proximal promoter, three potential Estrogen Response Elements (ERE) have been identified (Grimaldi et al., 2012). In fact, *in vitro*, estrogen receptor (ER) modulates FAAH gene expression independent of their ligands (Waleh et al., 2002). Following with this line, Grimaldi and coworkers (Grimaldi et al., 2013) showed that in Sertoli cells, the presence of estradiol (E_2) increases the mRNA levels of *faah* as well as the

FAAH enzymatic activity; progesterone upregulates FAAH activity in human lymphocytes (Maccarrone et al., 2004); E_2 regulates the expression of CB1 in brain (Riebe et al., 2010); FAAH has been reported to be down-regulated by sex hormones in rat uterus (Mauro Maccarrone et al., 2000) and acute exposition to E_2 in zebrafish altered the ECS in liver and brain (Martella et al., 2016).

Then, a clear interplay between these two systems has been described, hence, chemicals possessing estrogen-like (or anti-estrogen-like) or/and androgen-like (or anti-androgen-like) activity might induce alterations in the ECS.

b) THE ENDOCRINE DISRUPTOR CHEMICALS (EDC)

The International Programme on Chemical Safety (IPCS), a programme from the World Health Organization (WHO) defined an endocrine disrupting chemicals (EDCs) as: "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an impact organism, or its progenies, or (sub) populations" (World Health Organization, 2002).

The EDCs often disrupt the endocrine system by mimicking or blocking natural hormones, in fact, the EDCs can act:

- On the receptor protein complex, binding the receptors. Originally, the EDCs were thought to exert their actions through the nuclear hormone receptors, such as estrogen or androgen receptors. Nowadays, the scientific community showed that their actions are much broader; hence, EDCs can act also via non-nuclear steroid receptors, non-steroid receptors or orphan receptors.
- On the enzymatic pathways that control the biosynthesis and metabolism of steroids, altering the action of enzymes involved in the steroidogenesis. (Diamanti-Kandarakis et al., 2009).

However, a hormone receptor possesses a high affinity for its natural ligand, and usually, the EDC molecule has a lower affinity for, although, not lower potency. In this context, the EDC can act at very low dose, in fact, EDCs are characterized for producing non-linear responses, commonly observed in a U-shaped or inverted-U-shaped dose-response curve, namely "non-monotonic dose-response curve", where the strongest responses are exerted by the lowest or highest concentrations of the EDCs.

Since the endocrine system exert a critical role in many physiological and biological functions throughout lifespan, alterations induced by the activity of the EDCs can lead to pathological conditions, in fact, data from human, animal and *in vitro* studies have generated considerable evidences linking the EDC exposure to health disorders (Gore et al., 2014). As mentioned above, the EDC can act throughout organism lifetime and recently, it has been demonstrated that the EDCs can induce transgenerational effects passing from parents to the subsequent generations, modifying factors that regulate gene expression such as DNA methylation or histone modifications (Santangeli et al., 2017a, 2016)

Briefly, the principal disorders induced by EDC can be summarized in (Gore et al., 2014):

- Neurological and behavioral disorders.
- Obesity, metabolic dysfunction and related disorders.
- Reproductive disorders.
- Cancers.
- Other disorders (*i.e.* immune and inflammatory effects).

Unfortunately, the group of chemicals identified as EDCs is highly heterogenous and includes, among others, chemicals as polychlorinated biphenyls (PBC), polybrominated biphenyls (PBB), plastic additives (phthalates, bisphenols),

pesticides, fungicides or pharmaceutical products. Consequently, they are present in a large range of items and can be found in many common products, as plastic bottles, food cans, detergents, paints, sealants, cosmetics, medical devices or toys.

Indeed, we are going to focus in plastic additives which are man-made substances added into the polymeric matrix of plastic to increase their usefulness or provide them specific properties, as color or to protect the polymer from the degrading effects of light, heat or bacteria.

Among all the types of plastic additives existing, emphasis will be placed on the group of **plasticizers**. Plasticizers, are high-molecular-weight monomeric liquids with a high boiling point added to vinyl and cellulosic plastic to improve their flexibility and softness. Usually, their concentrations range around the 20-50% or more of the total of the plastic composition (Deanin, 1975), for instance, the group of phthalates constitutes up to 50% of total weight of PVC plastic (Oehlmann et al., 2009).

The European plastic manufacture association (Plastic Europe, 2015) estimated that the global production of plastic in 2015 was about 322 million tons, exponentially ramping from 1950 when the production was estimated around 1.5 million. Extraordinary is the case of Asia which account for more than 49% of worldwide production. In Europe, plastic production reached the 58 million of tons in 2014 (Plastic Europe, 2015), where 25.8 million tons of post-consumer plastic ended as waste, and only 17.9 million tons were recovered. Concomitantly with the increase of the production of plastic, the industry of plastic additives was estimated to increase with an annual rate of 4.4% during the period of 2013 – 2020 (Additives for polymers, 2014)

On this regard, Rochman and colleagues (Rochman et al., 2013a) estimated that if current consumption of plastic continues, the planet will hold another 33 billion

tons of plastic by 2050. Furthermore, the group of Jambeck (Jambeck et al., 2015) estimated that in 2010, from 4.8 to 12.7 million tons entered to the oceans. Thus, the ubiquity of plastic waste in the environment, and for instance, some EDC as primary constituent of the plastic items, turned into a health and an environmental worldwide problem. In fact, such problematic is becoming alarming for wildlife, in particular for the aquatic organisms, where a large part of plastic debris and sewage effluents ends up. In fact, the aquatic organisms may be not exclusively exposed to EDC via surrounding water, but by the accidental ingestions of plastic debris. Indeed, studies highlighting the ingestion of plastic debris by aquatic organisms have been lately increasing. It is remarkable a report done by the Food and Agriculture Organization of the United Nations (FAO) where plastic ingestion has been reported in the stomach of over 220 different marine species, 58% of which are classified as human target species (FAO, 2017). Indeed, this is not an exclusive problematic of teleosts (Lusher et al., 2013; Neves et al., 2015; Romeo et al., 2015), but seabirds (Codina-García et al., 2013), sea turtles (Lazar and Gračan, 2011) and marine mammals among others (Baulch and Perry, 2014; Denuncio et al., 2011). The risk of plastic ingestion is not just the physical harm produced by the plastic item but also because the plastic debris can concentrate other persistent chemicals diluted in the water as well as, may leach plastic additives and thus, introducing potential hazardous chemical to the biota after plastics are ingested (Rochman et al., 2013b).

Taking in account the aforementioned points, among the list of chemicals used as plasticizers, in the present thesis we are going to focus in two with special relevancy: the Di-isononyl phthalate (DiNP) and the Bisphenol A (BPA).

Di-isononyl phthalate (DiNP)

DiNP belongs to the family of high molecular weight phthalates for its long backbone carbon chain. DiNP is mainly used as a general-purpose plasticizer and it

is manufactured by esterifying phthalic anhydride and isononanol, which is composed of two branched alcohol isomers (C9).

About the 95% of DiNP is used for PVC applications and the other 5% for non-PVC ones. Due to its durability, fluidity and low volatility, DiNP turned into a model plasticizer and an excellent alternative for the most of di(2-ethylhexyl) phthalate (DEHP) uses, excepting for medical devices (European Chemicals Agency, 2012), after the Council Directive 67/548/EEC classified DEHP as toxic for reproduction. Consequently, the manufacture of DiNP has been increasing constantly since 1994 while the manufacture of DEHP has been decreasing.

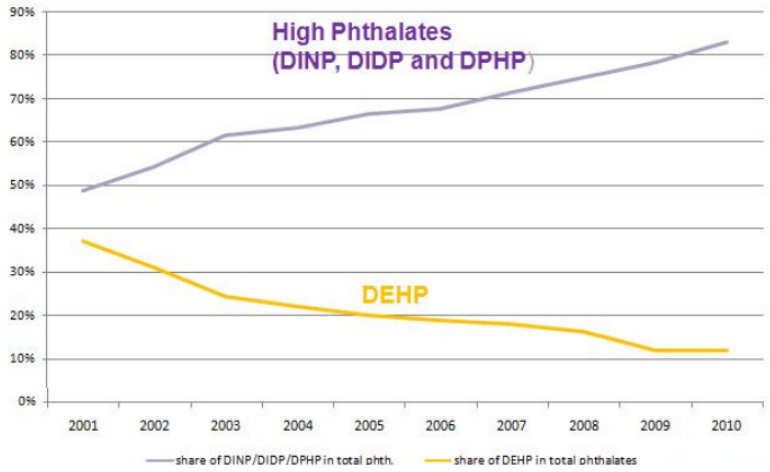

Figure 2. Percentage of phtahaltes sales in Europe (ECPI, 2011).

Noteworthy, DiNP, DIDP (Diisodecyl phthalate) and DPHP (Di(2-propylheptyl) phthalate) represents the 65% of the overall consumption of plasticizers in Europe and the 83% of the total sales of phthalates in Europe (European Council for Plasticisers and Intermediates, 2015). Anyway, restrictions for the uses of DiNP in toys and childcare articles existed, being mandatory not exceeding the presence of DiNP more than 0.1% (Directive 2005/84/EC). In addition, the European Food Safety Authority (EFSA) agreed to use the NOAEL (no-observed-adverse-effect-

level) of 15mg/kg/day and to establish a Tolerable Daily Intake (TDI) of 0.15 mg/kg body weight for human intake

DiNP can be found in the aquatic environments (Joel D Blair et al., 2009; Cheryl E. Mackintosh et al., 2004; Oehlmann et al., 2009), specifically, in water surface up to 0.7 µg/L (European Commission, 2003). DiNP possesses low water solubility and it is readily biodegradable (European Commission, 2003) with a short half-life. Rapidly metabolized, absorbed and excreted, Mckee and coworkers (McKee et al., 2002) affirmed that DiNP does not persist or accumulate in any organ; controversially, DiNP seems to be bioavailable in the environment and has been detected in wildlife organism tissues (Mackintosh et al., 2004). Even the high rate of excretion, the problematic of the DiNP might be the chronic exposure of wildlife. Indeed, experimental chronic exposure to high dosage of DiNP has been associated to:

- Effects in rat development (Boberg et al., 2011; Gray et al., 2000).
- Effects in male fertility in rats (Boberg et al., 2011; Kwack et al., 2010) and induction of several testosterone metabolites in male Japanese medaka (Patyna et al., 2006).
- Induction of hepatic and renal injuries (ECHA, 2013; Kaufmann et al., 2002; Ma et al., 2014; Moore MR, 1998; Smith et al., 2000; Valles et al., 2003; Wittassek and Angerer, 2008) and increase of liver weight (Kaufmann et al., 2002; Kwack et al., 2010). Indeed, McKee reported that liver and kidneys are the tissues where DiNP is primarily found after DiNP intake, being the most sensitive organs and targets for DiNP exposure (ECHA, 2013; Ma et al., 2014).

In addition, brief exposure to DiNP with high dosages during the perinatal period induced developmental impairments in the rat offspring (Borch et al., 2004; LEE et al., 2006; Masutomi et al., 2003).

Finally, after a carefully revision of the literature, the European Chemicals Agency in the "evaluation of new scientific evidence concerning DiNP" report concluded that DiNP possess anti-androgenic potency (ECHA, 2013). Furthermore, DiNP has been reported to possess weak estrogenic activity (Harris et al., 1997).

It is remarkable that most of the literature found regarding the effects of DiNP is based in studies using high dosages, being undescribed the consequences at environmentally relevant concentrations.

Bisphenol A (BPA)

First synthesized in 1891 and introduced in plastic manufacture around 1950 (Vogel, 2009), BPA is an organic compound composed with two phenol rings connected by a methyl bridge. BPA is a chemical intermediate used primarily (90%) for the production of polycarbonate products and epoxy resins and added in food and beverage containers, among other applications. Lesser amounts are used to produce fire retardants, antioxidants or stabilizers for the manufacture of PVC (Staples et al., 2002). Anyhow, its global production was predicted to exceed 5.4 million metric tons by 2015 (Market Publishers Ltd, 2014). Following the growing trend of plastic production, the total BPA world consumption is expected to grow at an annual rate of 3% in the period 2015 – 2020 (IHS Markit, 2016).

BPA is a moderately water-soluble compound (Staples et al., 1998), and as DiNP, is readily biodegradable, with a half-life of 0.5 – 3 days in aquatic ecosystems (Klecka et al., 2001).

BPA has been the subject of a considerable amount of studies due to its elevated applications and its ubiquity; indeed, BPA is used in so many common products that its exposure is thought to be nearly continual. For instance, BPA has been found in all environment compartments (soil, air, water) (Corrales et al., 2015). Specifically, in the aquatic environment, BPA in entering by landfills leachates, sewage effluents

(Oehlmann et al., 2008; Staples et al., 2002) and from migration of BPA-based products. Concentration of BPA in the aquatic systems can vary widely, depending on the sampling location, depth of water column and even among salt or freshwater, but the studies set up an estimation of the levels of BPA from 0.0005 to 8 µg/L (Wright-Walters et al., 2011) or up to 12 and 21 µg/L (Crain et al., 2007; Flint et al., 2012).

BPA was firstly reported to exert estrogenic activity by Dodd and Lawson in the thirties (Dodds and Lawson, 1936). In 1993, the team of Krishnan (Krishnan et al., 1993) determined that BPA was leaching from polycarbonate flask after autoclaving affirming that such leachate may affect further experimentations and reporting the estrogenic activity of BPA. In this line, BPA has been described to possess weak affinity for vertebrate receptor (Bolger et al., 1998) and as anti-androgenic chemical (Lee et al., 2003).

Nevertheless, the continuous presence of BPA raises the question whether environmental concentrations of BPA may have an impact in fish and wildlife organisms. Staples and collaborators (Staples et al., 2002) provided an exhaustive review of the chronic effects of BPA in aquatic organisms through a hazard assessment using ecologically relevant endpoints as growth or reproductive success. In their study, they concluded that for teleost, at the typical BPA surface water concentration, BPA is unlikely to cause adverse effects, evidencing detrimental effects with chronic exposure at concentrations of 160 µg/L or higher. However, since then, several studies have been published, showing that the aquatic environment is not sufficiently protected from BPA adverse effects at the established environmental concentrations (Wright-Walters et al., 2011), contrary to the affirmations of Staples et al., (2002). Actually, BPA is reported to induce alterations at low concentrations. For instance, the presence of ovotestes has been described in Japanese medaka at the concentration of 10 µg/L (Metcalfe et al., 2001); inhibition of the sword in swordtails male fish at 0.2, 2 and 20 µg/L (Kwak et

al., 2001); alterations in the zebrafish ovary and epigenome at 5, 10 and 20 µg/L (Santangeli et al., 2017a, 2016); reproductive impairment in several fish species at environmental concentrations (see Oehlmann et al., 2009 for review species and BPA concentrations). In addition, an extended bibliography of low-dose effects of BPA are available for murine models (vom Saal and Hughes, 2005) no reported here as mice and rats are not the experimental models for the present PhD project.

c) THE TELEOST MODELS

Two fish models were used to carry the experimentations in the present Doctorate project:

Wild-type Zebrafish (*Danio rerio*)

The cyprinid teleost zebrafish (*Danio rerio*) turns into an ideal model organism for environmental monitoring (Dai et al., 2014) due to its sensitivity to different contaminants, such as heavy metals, EDCs or organic pollutants. In the case of the EDCs, zebrafish can be used not only to evaluate the concentrations of the pollutants in the aquatic environment but also to assess their toxic effects. Further benefits are added to the use of such specie as model for ecotoxicological studies. For instance, zebrafish has a fully-sequenced genome; ease rearing conditions; short life-cycle reaching the sexual maturity in few months; high fecundity rate in captivity; small size; low cost husbandry; the possibility to obtain transgenic and mutant strains (Hill et al., 2005; Bopp et al., 2006) and the availability of a complete database (https://zfin.org/). However, additional benefits should be described when embryo or larvae are used, but it will be omitted as they are not the stage of zebrafish used for this Doctorate project. Actually, early stages of zebrafish are a very interesting model due to initially, zebrafish are born as females to become males after the ovarian regression and the development of the testes.

So, in the last years, zebrafish turned into one of the preferred fish species used in tests concerning environmental effects (Bopp et al., 2006) and several protocols and guidelines for the ecotoxicity assessment have been developed in the last years from ISO (International Organization for Standardization) and OECD (Organization of Economic Co-operation and development) for zebrafish experimentations.

Gilthead sea bream (*Sparus aurata*)

S. aurata possess an interesting biology which allow this specie to be a stimulating model for ecotoxicological studies due to sea bream is a protandrous hermaphrodite seasonal breeder specie. In fact, before the fish invert to female, gilthead sea bream possesses ovoestis, which during the reproductive season, the testicular part increases in size and the spermatogenesis takes place, while the ovarian part remains having previtellogenic oocytes. After the reproductive season (August – September), the two sexual components of the ovotestis may be equal. Then, the specimens can undergo sex reversal to become female, what in captivity is conditioned by social and hormonal factors and occurred more prematurely than in the wild, becoming the 80% of them into female (Zohar et al., 1978). At that point, the individuals begin the vitellogenesis increasing the ovarian part while the testicular regresses. On the other hand, the individuals which remain males initiate the spermatogenesis and the testicular part increases while the ovarian regresses. The gonad produces sperm during the spawning season, though the ovarian part does not disappear, it reduces the size and contains only primary oocytes.

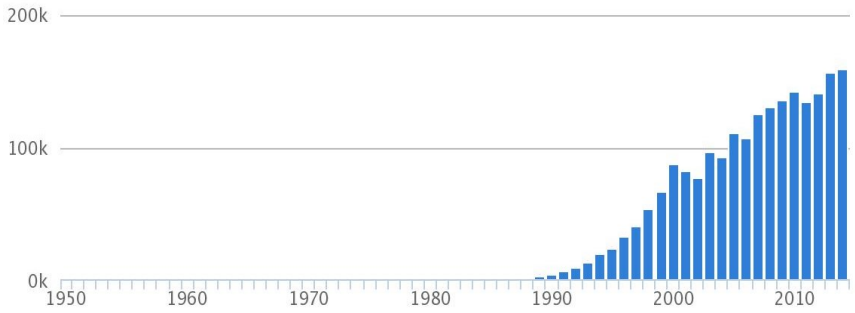

Figure 3. Global aquaculture production (tons) of *Sparus aruata*. Source: FAO FishStat.

In addition, gilthead sea bream is among the top ten marine farmed species (Teletchea, 2015) which its harvesting has been increasing from last decades, being a valuable and important economic resource. The European Union is the biggest producer worldwide, where Greece is by far one of the largest producer followed by Turkey, Spain and Italy (FAO, 2015). The success of the gilthead sea bream production falls on the high adaptability to intensive rearing conditions which can reach, on average, the commercial size after one year and a half.

Thus, the present thesis has been organized in five chapters regarding the issues previously mentioned in the introduction in order to assess whether **the two EDCs may induce alterations on the ECS**:

1. The effects of Di-isononyl phthalate exposure on the liver and brain of adult female zebrafish
2. The effects of Bisphenol A exposure on the liver and brain of adult female zebrafish.
3. The effects of Di-isononyl phthalate exposure on the gonads (male and female) of zebrafish.
4. The effects of Di-isononyl phthalate and Bisphenol A exposure on the liver and brain of adult male gilthead sea bream.
5. The effects of Di-isononyl phthalate exposure on the gonads and plasma steroids of adult male gilthead sea bream

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GENERAL OBJECTIVES

The present thesis has as a general aim the evaluation of the effects of two endocrine disruptors chemicals (EDCs), the Bisphenol A (BPA) and the di-isononyl phthalate (DiNP), on the endocannabinoid system (ECS), recently indicated as a new target for EDC's.

Specifically, the main goals are:

1) Given the increased use of DiNP as substitute of DEHP, the assessment of the effects of DiNP via water at environmentally relevant concentrations on the ECS at central (brain) and peripheral level (liver and gonads) in adult *Danio rerio* and the possible consequences in the hepatic lipid metabolism and the reproductive performance.

2) The evaluation of the effects of one of the most manufactured and widespread plasticizers, as the BPA, administered via water at environmentally relevant concentrations, on the ECS (brain and liver) and on the appetite signaling of adult *Danio rerio* female.

3) The determination of the effects of chronic exposure to DiNP and BPA via diet was studied in the central and hepatic ECS, in the appetite regulation and in the hepatic lipid metabolism in adult male *Sparus aurata*.

4) The effects of chronic exposure to DiNP via diet in the ECS at gonadal level and the subsequent reproductive performance, including sperm quality, sex hormone concentration in plasma and gonadal morphology in adult male *Sparus aurata*.

CHAPTER 1

Effects of Di-isononyl phthalate on the liver and brain of zebrafish

Dose – specific effects of Di-isononyl phthalate on the endocannabinoid system and on liver of female zebrafish

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Abbreviated Title: Effects of DiNP on endocannabinoid system.

Key words: DiNP, endocannabinoids, liver, zebrafish.

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ABSTRACT

Phthalates, used as plasticizers, have become a ubiquitous contaminant and have been reported for their potential to induce toxicity in living organisms. Among them, the Di-isononyl phthalate (DiNP) has been recently used to replace the bis(2-ethylhexyl) phthalate (DEHP). Nowadays, there is evidence that DiNP is an Endocrine Disrupting Chemical (EDC), however little is known about its effects on the Endocannabinoid System (ECS) and lipid metabolism. Hence, the aim of our study was to investigate the effects of DiNP on the ECS in zebrafish liver and brain and on hepatic lipid storage. To do so, adult female zebrafish were exposed to three concentrations (0.42 μg/L; 4.2 μg/L; 42 μg/L) of DiNP via water for 3 weeks. Afterwards, we investigated transcript levels for genes involved in the ECS of brain and liver as well as liver histology and image analysis, Fourier-Transform Infrared Spectroscopy (FT-IR) imaging, and measurement of EC levels. Our results demonstrate that DiNP upregulates orexigenic signals and causes hepatosteatosis together with deregulation of peripheral ECS and lipid metabolism. A decrease in the levels of ECS components at the central level was observed after exposure to the highest DiNP concentration tested. These findings suggest that replacement of DEHP with DiNP should be considered with caution because of observed adverse DiNP effects on aquatic organisms.

PRÉCIS

Female zebrafish treated with Di-isononyl phthalate showed fatty liver and deregulation of the endocannabinoid system, appetite signals and lipid metabolism.

INTRODUCTION

Phthalates are commonly used as general purpose plasticizers; for their specific chemicals properties, they are found in a large variety of items such as perfumes, food packaging, food containers or adhesives among others, with a manufacture of one million tons per year in Europe (European Chemicals Agency, 2010). Nowadays, phthalates constituted 78% of plasticizer demand in 2012 according to IHS-Markit (IHS-Markit, n.d.), however, there is evidence that phthalates can leach from plastics and be released into different environmental compartments due to non-covalent interaction with the matrix (Schettler, 2006). Hence, exposure to phthalates may occur directly by using phthalate-containing plastic devices or following exposure to a polluted environment (Giulivo et al., 2016). As a result, phthalates are ubiquitous anthropogenic contaminants which are reasonably abundant and known to have endocrine disrupting ability [4,5]. According to recent studies, phthalates are lipophilic and tend to accumulate in the adipose tissue (Chiang et al., 2016), although they are rapidly metabolized and have relatively short biologic half-life (Anderson et al., 2001; Koch et al., 2005). Nevertheless, their ubiquitous presence in the consumer products and environment can lead to chronic population exposure (Frederiksen et al., 2007; Fujii et al., 2003; Heudorf et al., 2007; Högberg et al., 2008; Latini et al., 2003; Schettler, 2006). Up to date, several studies have reported the endocrine-disrupting properties of phthalates, not only in humans, where they are linked to health disorders (Katsikantami et al., 2016; Meeker et al., 2009), but also in a wide range of aquatic and terrestrial

organisms (Carnevali et al., 2010; Golshan et al., 2015; Maradonna et al., 2013; Migliarini et al., 2011; Oehlmann et al., 2008).

Among the family of high molecular weight phthalates, Di-isononyl phthalate or DiNP (CAS No 28553-12-0) has replaced the widely used chemical di(2-ethylhexyl) phthalate (DEHP), which is known for its adverse consequences in humans and wildlife (Maradonna et al., 2013). DiNP has become one of the dominating alternatives of DEHP due to their similar properties (ExxonMobil Petroleum and Chemical B.V.B.A, 2014), except in medical products (European Chemicals Agency, 2010). The globally forecasted growth in DiNP consumption between 2012 and 2018 represents nearly 5 times the current DEHP use in Europe (IHS-Markit, n.d.), where the family of high molecular weight phthalates (DiNP, Diisodecyl phthalate (DIDP), Dipropylheptyl phthalate (DPHP)) holds 57% share of the plasticizers market (IHS-Markit, n.d.).

Ninety-five percent of the manufactured DiNP is mainly used in PVC applications and the other 5% is used in inks, adhesives, sealants, paints and lubricants (European Council for Plasticisers and Intermediates, 2015). DiNP is also used widely in toys, food packaging, drinking straws and gloves, among other consumable products. Like other members of the phthalate family, DiNP represents an ecological problem due to its weak bonding to the matrix and ease of release to the environment (Fujii et al., 2003). Thus, DiNP can leach into the food stored in plastics and be released in the environment (I. for H. and C. P. E. C. B. European Commission, 2003). Indeed, DiNP can be detected in frozen meals, oil, fat and breads (Environment Canada, 2015). According to a European Union sponsored study, DiNP exposures for adults were approximately 11µg/kg/day, whereas exposures in children, from sources other than toys, were approximately 50µg/kg/day (I. for H. and C. P. E. C. B. European Commission, 2003). While both estimates fall below the Tolerable Daily Intake for DiNP, exposures of children could potentially reach higher via mouthing activities. So, since January 2014, DiNP

use was restricted in toys and items for children under three years of age (Directive 2005/84/EC). In addition, the U.S and Canada have implemented similar restrictions on the use of DiNP in toys and childcare articles that can be placed in the mouth, thus limiting to 0.1% the presence of DiNP.

Fortunatly, like other phthalates, DiNP accumulation in tissues is not persistent (McKee et al., 2002). DiNP appears in the liver and kidney shortly after ingestion, followed by catabolism and excretion via urine with a half-time of 7 hours. However, as for other phthalates, increased use of DiNP may lead to chronic exposure of organisms (Environment Canada, 2015). In this context, DiNP was detected in 72% of the species sampled by Mackintosh and collaborators, confirming that the substance is bioavailable to aquatic biota (Cheryl E Mackintosh et al., 2004). Liver is one of the most sensitive organs following chronic exposure to DiNP (United States; Consumer Product Safety Commission, 2010; Wittassek and Angerer, 2008) with possible toxicological consequences, based on a number of studies including: 1) hepatic alterations in SV129 wild type mice after a week of exposure(Valles et al., 2003); and 2) non-neoplastic lesions, such as necrosis or spongiosis hepatis (McKee et al., 2002) and hepatocellular enlargement among others in Fischer 344 rats exposed for two years (Moore MR, 1998). In addition, DiNP has also been shown to exhibit peroxisome proliferator properties (Kaufmann et al., 2002; Valles et al., 2003).

To date, several studies in mice have highlighted the toxicity of DiNP, not only in the liver, but also in the brain. DiNP was shown to cause injury in hippocampus pyramidal brain cells as well as oxidative stress, leading to impaired cognitive abilities and anxiety-like behaviour in exposed mice (Ma et al., 2015; Peng, 2015). In addition to the myriad of effects of DiNP, this compound was found to act as an estrogenic chemical in zebrafish and medaka (Chen et al., 2014) and cause abnormalities in androgen-dependent tissues in other species (Gray et al., 2000; Howdeshell et al., 2008; Lee and Koo, 2007). For instance, in rats exposed to DiNP,

inhibitory effects on fetal testicular testosterone production and content, associated with the tendency to reduce plasma testosterone levels, were reported (Borch et al., 2004).

Recently, the ECS has emerged as a key player in the regulation of energy homeostasis and food intake (Cota, 2007; Piccinetti et al., 2010b; Silvestri et al., 2011) and a metabolic mediator from the central nervous system to the periphery, and *vice versa* (Piccinetti et al., 2010b). The ECS is comprised of: two G-protein-coupled receptors: cannabinoid receptor type 1 (CB1) and type 2 (CB2); two endogenous ligands: the endocannabinoids (EC) *N*-arachidononyl-ethanolamine (Anandamide or AEA) and 2-arachidononyl-glycerol (2-AG); and the enzymes regulating the levels of ECs. The metabolic enzymes are the *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and sn-1-diacylglycerol lipase (DAGLα) for the biosynthesis of AEA and 2-AG, respectively. AEA is mainly catabolized by the fatty acid amide hydrolase (FAAH), and 2-AG by monoacylglycerol lipase (MAGL), into arachidonic acid plus ethanolamine and glycerol, respectively. Some studies have shown that the dysregulation of the components of the ECS is associated with several metabolic disorders compromising the well-being of the organisms. For example, in the liver, which is one of the major organs involved in energy homeostasis, the ECS may be a contributing factor in inducing steatotic liver through disruption of hepatic lipogenic and lipolytic pathways (Silvestri et al., 2011). The activation of CB1 in hepatocytes induces the synthesis of lipogenic enzymes and increases *de novo* fatty acid production (Cinar et al., 2014; Osei-Hyiaman et al., 2005) while CB2 activation, instead, reduces adipose tissue and liver inflammation (Deveaux et al., 2009).

In addition to the peripheral control, an important role is exerted by the ECS at the central level (Di Marzo and Matias, 2005), where CB1 is the most abundant G-protein-coupled receptor in the mammalian brain (Christopher S. Breivogel and Steven R. Childers, 1998), and endocannabinoids work as rapidly as produced and

quickly deactivated (Cota, 2007). It is well established that CB1 receptor agonists or antagonists strongly affect feeding behaviours (Cota, 2007), and activation of CB1 leads to stimulation of food intake (Piccinetti et al., 2010b), while a reduction in the activity by an antagonist leads to reduce food consumption (Di Marzo and Matias, 2005).

Nevertheless, in spite of the DiNP potential noxious effects on lipid metabolism and energy homeostasis as previous aforementioned, to date, no reports are available on how the plastic component DiNP may interfere with the ECS. Thus, the main objective of this study was to evaluate the effect of this relatively new plasticizer administered via water on *Danio rerio* metabolism. For the accomplishment of our objectives, the expression of several key genes of lipid metabolism, ECS and appetite control have been analyzed in the liver and brain of zebrafish exposed for 3 weeks to environmentally relevant concentrations of DiNP (Bergé et al., 2013; I. for H. and C. P. E. C. B. European Commission, 2003; Staples et al., 1997). In addition, we measured ECS in the liver and brain, and investigated histology as well as biochemical composition of the liver by Fourier-Transform Infrared Spectroscopy imaging (FT-IR).

MATERIALS & METHODS

Animals and DiNP treatment.

The experiments and the analysis were carried out exclusively with adult female zebrafish (*Danio rerio* wild-type strain) in order to contain the number of specimens to be treated and sacrificed as well as to avoid possible bias related to gender difference of EDC by acting through sex specific pathways. The specimens used were one year old females, sexually mature.

A three-week period treatment with DiNP was conducted in duplicate, for each replicate, a final housing density of 25 female zebrafish were placed per tank

according to protocols and procedures approved by the University of Calgary Animal Care Committee (protocol # AC15-0183). Three nominal concentrations of DiNP [42 µg/L (10^{-7}M); 4.2 µg/L (10^{-8}M); 0.42 µg/L (10^{-9}M) (99% purity, Sigma-Aldrich)] were used in 100-L aquaria supplied with oxygenated water and controlled conditions (28.0 ± 0.5˚C under a 14/10 hours of light/dark period). The specimens were fed twice per day with a 1:1 mixture of adult zebrafish complete diet (Zeigler Bros., Inc.) and live brine shrimp nauplii, Artemia cysts.

Due to the lack of data regarding the concentrations of DiNP in water, nominal doses administrated to zebrafish were chosen after carefully review of previously published papers (Bergé et al., 2013; Staples et al., 1997) and taking into account the predicted DiNP concentration in water surface by the European Community, which is 0.7 µg/L (I. for H. and C. P. E. C. B. European Commission, 2003). A molecular weight of 420 g/mol and its multiples were used for the calculation of DiNP molar concentrations.

After twenty-one days of treatment, fish were euthanized with buffered MS-222 (3-aminobenzoic acid ethyl ester) according to University of Calgary Animal Care Committee (protocol # AC15-0183) and sampled at 7-9 a.m. to avoid any possible circadian variability. Livers and brains from each experimental group were removed and immediately frozen on dry ice and transported to the analytical laboratory where they were stored at -80ºC for FT-IR, RNA extraction and Endocannabinoids assays. Livers were fixed in Bouin solution for histology.

RNA extraction and cDNA synthesis

Total RNA was isolated from liver and brain with RNAzol solution (Sigma Aldrich) according to the manufacturer's instructions and as described previously (Santangeli et al., 2016). Final RNA concentrations were determined with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific), while RNA integrity was verified by red staining of 28S and 18S ribosomal RNA bands on a 1% agarose

gel. Then 3 µg total RNA was used for cDNAs synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy) and kept at −20 °C until use.

Real-Time qPCR

The relative quantification of gene expression was performed with the SYBR green method in an iQ5 Multicolor Real-Time PCR Detection system (Bio Rad) as described previously (Santangeli et al., 2016). Primer list is reported in table 1 and used at a final concentration of 10 pmol/µL.

Two different genes, namely *Arp* (Acidic Ribosomal Phosphoprotein) and *Rpl13a* (Ribosomal protein L13a), were used as internal controls (Roesner et al., 2006; Tang et al., 2007) to minimize variations in mRNA and cDNA quantity and quality. Reference genes were chosen because the mRNA levels did not vary between experimental treatments. No amplification product was observed in the negative control (absence of template). Specificity of primer and the absence of primer-dimer formation was indicated by a single peak in the dissociation curve obtained at the end of the amplification cycle.

Data were analysed using iQ5 Optical System version 2.1 (Bio-Rad). The quantification method was based on a ΔΔCt calculation implemented with the Pfaffl equation. Data were generated in duplicate from 5 biological replicates and expressed as relative mRNA levels (Unscaled abundance).

Measurement of endocannabinoids (AEA, 2-AG) and endocannabinoid-like mediators (PEA, OEA) in the brain and liver.

The extraction, purification and quantification of ECs from tissues were performed as previously described(Vincenzo Di Marzo et al., 2001). Briefly, the tissues were dounce-homogenized and extracted with chloroform/methanol/Tris–HCl 50 mmol l–1 pH 7.5 (2:1:1, v/v) containing internal standards ([2H]8 AEA 5 pmol; [2H]5 2-AG, [2H]5 PEA, and [2H]4 OEA 50 pmol each). The lipid-containing organic phase was dried down, weighed, and prepurified by open-bed chromatography on

silica gel. Fractions were obtained by eluting the column with 99:1, 90:10, and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA, and OEA quantification by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry by using a Shimadzu high-performance liquid chromatography apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2020) quadruple mass spectrometry via a Shimadzu atmospheric pressure chemical ionization interface. The amounts of analyses in tissues quantified by isotope dilution with the abovementioned deuterated standards were expressed as picomoles per gram or milligram of wet tissue weight.

FAAH enzymatic activity

A pool of 5 livers were homogenized at 4°C in 50mMTris-HCl buffer, pH 7.0, centrifuged at 800 g and the, the supernatant was collected and centrifuged at 10 000 g. Protein concentrations were measured by Bradford assay (Biorad). 70 µg of protein from liver membranes were incubated with [^{14}C] AEA (10,000cpm, 1.8M) in 50 mM Tris- HCl, pH9, for 30 minutes at 37°C. [^{14}C] Ethanolamine produced from [^{14}C] AEA hydrolysis was then extracted from the incubation mixture with 2 volumes of $CHCl_3$/CH_3OH (1:1 by volume) and the subsequent aqueous phase measured by scintillation counting in order to calculate FAAH activity. Assay performed in triplicates.

Liver Morphology

The liver samples (n=7/experimental group) were fixed in Bouin and prepared for histological examination using standard biological procedures. The fixed tissues were embedded in paraffin and sectioned (4 µm) with a microtome (Leitz 1512). Each liver was fully sectioned and processed for Mayer's hematoxylin-eosin staining and observed under a Zeiss Axioskop microscope. Microphotographies were captured using a high resolution digital camera (Canon EOS 6D).

Area covered with lipids vacuoles in the liver (ACVL%)

The estimate of hepatic lipid area was done according to the methodology of Papadakis et al. (2013) (Papadakis et al., 2013). For each liver, 5 microphotographs were obtained at ×100 magnification from different areas of the liver. A total number of 140 photographs were analyzed with ImageJ (https://imagej.nih.gov/ij/).

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR measurements were carried out by using a Bruker VERTEX 70 interferometer coupled with the Hyperion 3000 Vis-IR micro-scope and equipped with a liquid nitrogen cooled bidimensional focal plane array (FPA) detector (area size 64 × 64 pixels) as described previously (Gioacchini et al., 2014).

Statistical analysis

The statistical analyses on gene expression, ECs levels, FAAH enzymatic activity and FT-IR were performed with one-way ANOVA analyses of variance followed by *post hoc* Tukey's test. Data fulfilled the conditions for applying a parametric test, and log-normalization to homogenize the variance was used when needed. Confidence interval was set at 95% ($p < 0.05$). Results are presented as mean ± SD. Statistical analysis for hepatic histology were performed using also ANOVA but the results are expressed in percentage and reported as mean ± SD. All statistical procedures were run using *GraphPad Prism 6*.

RESULTS

DiNP alters central *cnr1* and *cnr2.* We first tested whether DiNP modulates zebrafish ECS at central level, including the genes encoding for the main receptors and the enzymes in charge of the synthesis and catabolism of the EC. Hence, the genes encoding CB1 and CB2 (i.e. *cnr1* and *cnr2*) in the brain were significantly

upregulated by the exposure of fish to the 0.42µg/L and 4.2µg/L (for *cnr1*), and 4.2µg/L and 42µg/L (for *cnr2*) of DiNP (Figure 1a). Regarding the genes encoding the ECS enzymes, the mRNA levels of *nape-pld* and *abdh4*, enzymes responsible of the synthesis of AEA, did not vary among experimental groups. Similarly, *faah*, gene encoding the enzyme which breaks AEA down into arachidonic acid (AA) and ethanolamine (EtNH2), were not affected by DiNP exposure. Likewise, exposure to DiNP did not alter transcript levels for *daglα* and *mgll*, enzymes that regulate the endocannabinoid ligand 2-AG. Finally, a significant decrease in *cox-2* transcript level which is involved in the conversion of the EC ligands AEA and 2-AG into prostamides and prostaglandin glycerol esters (inflammatory precursors) were induced following exposure to all concentrations of DiNP tested (Figure 1b).

DiNP upregulates appetite signals. In the line of the results previously described and since ECS is deeply involved in the regulation of food intake, the main signals for the appetite were studied in order to confirm if such EDC may induce alterations in the appetite regulation in the brain. Hence, the exposure to 0.42µg/L and 4.2µg/L DiNP resulted in a significant increase in the transcript levels for *npy*, which is an orexigenic neuropeptide with an important role in the modulation of food intake and obesity (Figure 2). Exposure to 4.2µg/L and 42µg/L DiNP significantly increased the mRNA level for *mc4r* which is a gene encoding another enzyme involved in the metabolic regulation of food intake in the central nervous system and finally, there was no change in the results of the qPCR for *lepr* gene which is an appetite molecular marker induced by leptin (satiety hormone).

DiNP modulates ECS peripheral signals. As described above, liver is one of the main DiNP targets, then, following the altered results at central level, the same ECS markers were also tested at hepatic level. Indeed, the exposure to DiNP increased the mRNA levels of the principal EC receptors, *cnr1* and *cnr2* in the liver in a dose-related manner. Transcript level for *pparα* was also increased following exposure with the 0.42 and 4.2 µg/L DiNP, while *pparγ* mRNA level was not affected (Figure

3a). Regarding the control of EC level, exposure to all concentrations of DiNP significantly decreased transcription of genes encoding the enzymes synthesizing AEA, including *nape-pld* and *abdh4*. However, the DiNP treatment did not affect the transcription of *faah* which encodes the enzyme hydrolyzing AEA, but significantly reduced the mRNA level for *cox-2*, catabolic enzyme at all concentrations tested (Figure 3b). On the other hand, DiNP exposure at lower concentrations (0.42 and 4.2 µg/L) increased the transcripts for *daglα* and *mgll* genes that encode 2-AG synthetic and hydrolytic enzymes, respectively.

DiNP induces *de novo* hepatic fatty acid synthesis and steatosis by modulation lipogenic and lipolytic gene expression. Ones established that DiNP induced alterations at ECS level, we want determine whether DiNP-induced hepatoesteatosis was associated with the ECS variations. We observed that DiNP exposure was without effect on *fasn* transcript level (enzyme that catalyze the synthesis of long-chain saturated fatty acids), but increased transcription level for *agpat4* (enzyme for *de novo* phospholipid biosynthesis) (Figure 4). As for the lipogenic enzymes, DiNP exposure decreased transcript for *dgat2* (enzyme involved in the final reaction of triglycerides synthesis), as well as *dgat2*, *acox-3* and *acat-2* (key enzymes of the β-oxidation of lipids and sterol synthesis), *hnf4α* (a major transcriptional regulator of many genes related to lipid homeostasis in the liver and a marker of liver condition), and *lepr* (leptin receptor) in the liver. In this organ, the level of leptin is directly correlated with the expression of its receptor (Cohen et al., 2005) and it is involved in energy homeostasis and accumulation of fat. We also performed histological analyses on the liver after we have seen the alteration of the molecular markers revealing severe lipid accumulation in the hepatic tissue of animals exposed to DiNP (Figure 5a). Cytosolic lipid accumulation was normally accompanied by a loss of hepatocyte shape and ultimately the hepatic architecture. These postulates are supported by the observed increase of the area of the liver covered by lipids following treatments (Figure 5b).

Effect of DiNP on EC and EC-like mediator levels in zebrafish brain and liver. Considering the molecular results at central and peripheral level regarding the ECS pathway, the levels of endocannabinoids (AEA and 2-AG) and endocannabinoids-like mediators Palmitoylethanolamide (PEA) and Oleoylethanolamine (OEA) were measured in zebrafish brains and livers after chronic exposure to DiNP. In the brain (Table 2), the 4.2 µg/L and 42 µg/L concentrations induced a 50% reduction of the most abundant EC, AEA, but did not affect 2-AG level. The levels of PEA and OEA were reduced by all the treatments, but only the highest concentrations of DiNP significantly decreased OEA levels. On the other hand, DiNP treatments generally increased AEA levels in the hepatic tissue (Table 3), although only the 4.2 µg/L and 42 µg/L concentrations were statistically significant. Accordingly, the activity of FAAH, the main catabolic enzyme of AEA, was reduced by all the treatments (Figure 6), however the levels of 2-AG remained unchanged. About the EC-like mediators, PEA level was significantly increased following exposure to 0.42 µg/L and 4.2 µg/L of DiNP, whereas the same doses reduced the OEA levels. Surprisingly, neither PEA nor OEA levels were affected following the exposure to the highest DiNP concentration.

DiNP modifies the biochemical composition of liver increasing lipidic content. In order to confirm the ability of DiNP to alter the lipid metabolism in the liver, we investigated whether DiNP can modify lipids by FT-IR analysis**.** Then, three randomly-selected areas were chosen among all liver sections to determine chemical composition. The spectra extracted from the chemical maps were integrated under 2990-2836 cm^{-1} (lipids), 1765-1723 cm^{-1} (COO groups, triglycerides) and 3027-2995 cm^{-1} (=CH groups) to investigate lipid metabolism changes. The sum of the integrated areas 2990–2836 cm^{-1} and 1765 – 950 cm^{-1} was indicative of the total cellular biomass of the selected area considered (*cell*). All the values indicative of hepatic fat characteristics were normalized to the *cell* value. These bands were chosen in order to quantitatively locate the presence and the characteristics of hepatic fat. Chemical maps integrated under lipids gave a visible

display with a false color scale (Figure 7a). The intensity of the signal associated with a specific band, provide information on both the presence and the localization of the molecular/chemical group and as we can see, the intensity increased concomitant with the doses of the phthalate applied. A quantitative analysis was also performed as it is shown in Figure 7b for lipids, where the quantity and quality of lipids were analyzed. All treatments modified the cell composition by increasing the amounts of lipids and among the lipids, the triglycerides. Regarding the characteristics of the lipids, surprisingly all the treatments increased the length of the aliphatic chain (CH_2/CH_3 ratio).

DISCUSSION

Recent reports demonstrated how certain environmental chemicals can induce obesity by promoting adipogenesis and disrupting some basic physiological functions (Grün and Blumberg, 2009; Lv et al., 2016). Among the myriad of chemicals exhibiting this effect (Grün and Blumberg, 2009), common plasticizers such as phthalates are noteworthy. Many studies have been published regarding the obesogenic activity and the promotion of metabolic disorders induced by different phthalates in humans (Legler et al., 2015; Stahlhut et al., 2007), rodents (Hao et al., 2012; Lv et al., 2016) and in the aquatic model, zebrafish; however, the literature about DiNP is still scarce.

Regarding the ECS, the cannabinergic pathway which is one of the major circuits for appetite and body weight regulation, would be a perfect candidate to test obesogenic property of pollutants (Arias Horcajadas, 2007; Grün and Blumberg, 2009). ECS deregulation has been mainly studied in humans and rodents. However, more recently using zebrafish as an experimental model, it was demonstrated that exposure to bisphenol A (BPA) increases hepatosteatosis by upregulating ECS signaling (Martella et al., 2016). And now, the present study provides information for the first time on adverse effects of environmentally relevant concentrations of

another EDC, DiNP, on metabolic abnormalities at the ECS level using a well-established approach. Alterations of ECS can in turn effect the regulation of food intake and energy homeostasis. Initially, it was thought that the control of energy intake by the ECS occurred mainly centrally via the regulation of several hypothalamic anorexic and orexigenic mediators. However, there is evidence that the ECS, particularly via activation of CB1 receptors, also play a role in controlling peripheral organs such as liver, involved in food intake and energy balance (Di Marzo and Matias, 2005).

At central level, the neuromodulatory role of endocannabinoids through CB1 receptors in brain areas controlling food intake is well established. Furthermore, the ECS may influence the expression and action of several hypothalamic anorexic and orexigenic mediators, such as NPY (Gamber et al., 2005). NPY is a well-studied potent orexigenic neuropeptide and a promoter of lipid storage (Yokobori et al., 2012). In the present study, we found an upregulation of the genes encoding for NPY and CB1, i.e. *npy* and *cnr1*, induced by the 0.42µg/L and 4.2µg/L concentrations of DiNP in the zebrafish brain, suggesting that NPY may require CB1 to exert its orexigenic effects, as previously demonstrated by Gamber (Gamber et al., 2005) in rats. Focusing on the results obtained here in the zebrafish brain, it appears that at lower concentrations (0.42 and 4.2 µg/L) DiNP may regulate appetite via CB1, whereas at higher concentrations it may do so via CB2 as it was demonstrated in chickens and mice (Emadi et al., 2011; Onaivi et al., 2008), although so far there has been no evidence that CB2 is involved in food intake in other species of mammals or fishes. We also found an increase of *mc4r* expression at the higher DiNP concentration, suggesting that the receptor encoded by this gene, which is strongly anorexigenic, may counteract the orexigenic effects of the pollutant. To gain further insight into the effects of DiNP on the brain, we determined mRNA levels of the leptin receptor gene, *lepr*. Leptin, produced in the liver of teleost fishes, binds to specific receptors in the brain where it reduces food intake by downregulating orexigenic factors, such as NPY (Wang et al., 1997). We

found unchanged levels of brain *lepr* expression, suggesting that the increase of orexigenic factors such as *cnr1*, *npy* and *cnr2* may not be associated with actions of this hormone in the zebrafish brain. However, we did not measure the circulating levels of leptin and hence cannot exclude its role in DiNP effects.

Following with the ECS, DiNP exposure reduced AEA level and was without effect on 2-AG in the brain, which is different from the observed increases in the expression of the main endocannabinoid receptor or the metabolic enzymes (*nape-pld, abdh4, faah, mgll, daglα*). Similarly discrepancies were observed following exposure with BPA by Martella (Martella et al., 2016) in zebrafish, where the brain concentrations of EC did not change, despite the upregulation of *cnr1* at the central level. The observed reduced level of EC-like compound, OEA, which is known to reduce food intake in rodents via activation of PPAR (Rodriguez de Fonseca et al., 2001; Schwartz et al., 2008) is consistent with the reported by Martella and colleagues (Martella et al., 2016) following BPA administration, suggesting that OEA could also play a role in the enhancement of appetite together with enhanced CB1 expression. Thus, taken together, these results lead us to the hypothesis that a negative feedback on ECS signaling might occur in the brain following contaminant-induced upregulation of CB1 (and CB2) levels.

At the peripheral level, all DiNP concentrations caused a dose-related rise of hepatic fat as evidenced by histological and FT-IR analysis. This is the first study describing the ability of DiNP to induce fatty liver in zebrafish. However, several papers reported the steatotic properties of many phthalates in zebrafish, such as diethyl phthalate (DEP) in zebrafish embryos (Kim et al., 2015) or dibutyl phthalate (DBP) in adults (Xu et al., 2014). It is well stablished that, at the hepatic level, the ECS may contribute to fatty liver through disruption of lipogenic and lipolytic pathways. For instance, it has been demonstrated that activation of CB1 by AEA induces steatosis in mice (Osei-Hyiaman et al., 2005; Ravinet Trillou et al., 2003), whereas CB1 pharmacological blockade or genetic inactivation in hepatocytes

reverses hepatic steatosis (Tam et al., 2010). In our study, hepatic *cnr1* expression was only upregulated by the highest DiNP concentration, although the transcripts for the other main endocannabinoid receptor, CB2 (*cnr2),* and the content of lipids were increased in all groups. Similar to obese mice, in which an increase of *Cnr2* mRNA levels was measured (Deveaux et al., 2009), in zebrafish *cnr2* also seems to be a biomarker for (rather than the cause of) steatosis. The observed increase in the hepatic level of AEA and the unchanged level of 2-AG in the groups exposed to DiNP are consistent with reported results in mice fed with high-fat diet and developing fatty liver (Osei-Hyiaman et al., 2005) and in zebrafish exposed to BPA (Martella et al., 2016), supporting the concept that increased AEA and, hence, CB1 activity, is one of the pathways for the appearance of steatosis.

The increase of hepatic AEA levels in the present study, however, was not correlated with changes in the expression of genes coding for its synthetic enzymes, *nape-pld* and *abdh4*, which were in fact down-regulated in the treated fish. On the other hand, there was a decrease in the activity of FAAH in the liver and a reduction in the transcript abundance of another endocannabinoid degrading enzyme, i.e. *cox-2* [61], following exposure to DiNP, that might explain the increased AEA levels. Also BPA-induced down-regulation of FAAH expression and/or activity was suggested as the cause of the enhancement of hepatic AEA levels (Martella et al., 2016). We hypothesize that increased level of AEA leads to a negative feedback mechanism leading to lower levels of *nape-pld* and *abdh4* expression to avoid further increase in the level of AEA in the liver. On the other hand, increases induced by DiNP in the expression of the major enzymes catalyzing 2-AG biosynthesis (DAGL□) and inactivation (MAGL) may in part be a compensatory mechanism minimizing change in hepatic 2-AG levels following exposure to the phthalate. Regarding the EC-like mediators, PEA and OEA are endogenous ligands of PPARα, and the latter compound can stimulate lipolysis (Osei-Hyiaman et al., 2005) via this nuclear receptor, independent of CB1 in the liver. Hence, the lower hepatic OEA levels found in DiNP-exposed zebrafish might explain the steatotic

condition, and the observed increase in *pparα* may be part of an adaptive response to minimize the consequences of elevated AEA and reduced level of OEA. On the other hand, the increased levels of hepatic PEA induced by DiNP might be related to its anti-inflammatory actions since high levels of this mediator have been found during inflammatory processes (Darmani et al., 2005) of which steatosis represents an example.

A considerable amount of studies suggest that PPARα agonists exert several effects in the rodent liver such us hepatomegaly, cell proliferation, peroxisome proliferation and glucose and lipid metabolism among others (Burri et al., 2010; Klaunig et al., 2003; Pawlak et al., 2015); such effects are not present in PPARα-null animals (Knauf et al., 2006; Valles et al., 2003). Interestingly, DiNP *per se* is considered a PPARα and PPARγ agonist in humans and rodents (Kaufmann et al., 2002; Ma et al., 2014; Valles et al., 2003) promoting β-oxidation of fatty acids (Schoonjans et al., 1996). The observed stimulatory effect of DiNP on *pparα* expression is in agreement with previous studies (see review by Grun and Blumberg (Grün and Blumberg, 2009)), and is consistent with the idea that this phthalate, like a number of others xenobiotics, may interact with PPARs, leading to disrupt adipogenesis and related transcriptional signaling.

However, DiNP did not alter exclusively the ECS, also affected several metabolic key markers as leptin receptor, *lepr,* in the liver. Different from its role at the central level, deficiency of peripheral leptin signaling can lead to increased storage of lipids in rodent liver (Huang et al., 2006) and hence participate in the hepatic steatosis observed in zebrafish. In addition, increased expression of *agpat-4*, encoding for an enzyme involved in the second step of *de novo* lipid biosynthesis, supports our hypothesis that DiNP disrupts the metabolic processes in zebrafish. In contrast, we observed a decrease in *dgat2* transcript level which is involved in the second step of the lipid biosynthesis. Commonly, during steatosis, *dgat2* is over-expressed; however, in *dgat*-deficient mice, the synthesis of triglycerides still occurs (Smith et

al., 2000) as in our study, where the abundance of triglycerides with respect to the total amount of lipids augmented following exposure to increasing concentrations of DiNP. It is interesting to note that in fungus *M. ramanniana*, DGAT2 possesses higher preference for medium-chain than long-chain substrates (Lardizabal et al., 2001). Consequently, another explanation for the drastic downregulation of this enzyme might lie in the higher availability of fatty acids with longer aliphatic chain, as shown in our study by FT-IR. A similar down-regulation was found for *acat2*, a gene encoding an enzyme related to the hepatic triglycerides metabolism. In this context, ACAT2 knock-out mice are protected against lipid accumulation and present lower levels of hepatic triglycerides (Alger et al., 2010). In the light of the results reported here, we therefore hypothesize that *dgat2* and *acat2* were downregulated probably as an adaptive response to the high level of steatosis, in order to mitigate the overproduction of triglycerides without affecting *fasn* expression.

Adding further evidence of the metabolic impairment induced by DiNP, two other markers for steatosis such as *hnf4α* and *acox-3* were drastically downregulated by the phthalate. The *hnf4α* is a major regulator of genes involved in the control of lipid homeostasis in liver (Hayhurst et al., 2001) and studies with *hnf4α* deficient livers revealed excessive lipid accumulation in mouse hepatocytes (Hayhurst et al., 2001; Martinez-Jimenez et al., 2010). Similarly, *acox-3* is involved in the β-oxidation of fatty acids and *acox-3* deficient mice showed hepatomegaly with steatosis (Fan et al., 1996) in agreement with our histological and biochemical results where all the treatments with DiNP induced an increase of lipids in the hepatic tissue. Therefore, unlike the alterations of *dgat2* and *acat2* expression, those induced by DiNP on *hnf4α* and *cox-3* expression might be among the causes, and not the consequences, of DiNP-induced steatosis.

In conclusion, the results obtained here and summarized in Figure 8, indicate that environmental concentrations of DiNP, a widely-used plasticizer, induce an

impairment of lipid metabolism, hepatic steatosis and a dysregulation of the central and peripheral ECS in zebrafish. As a consequence of the present study, industry and regulators should be more cautious about the use and release of DiNP in the environment. Our observations provide a basis for future studies on the mechanisms of action of DiNP which is now a common environmental contaminant and these findings would also be relevant to more accurate assessment of potential ecological impact of emerging pollutants in the aquatic ecosystems.

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TABLES & FIGURES

Table 1. Summary table of primers used for qPCR analysis with their corresponding gene target name, annealing temperature and accession numbers. *arp*: acidic ribosomal protein; *rpl13a:* Ribosomal protein L13a; *cnr1:* endocannabinoid receptor type 1; *cnr2:* endocannabinoid receptor type 2; *daglα:* diacylglycerol lipase alpha; *mgll:* monoglyceride lipase; *nape-pld*: N-acyl Phosphatidylethanolamine Phospholipase D; *abdh4*: alfa/beta-hydrolase domain 4; *faah:* fatty acid amide hydrolase; *cox-2*: cyclooxygenase 2; *pparα*: peroxisome proliferator activated receptors α ; *pparγ:* peroxisome proliferator-activated receptors γ; *fasn:* fatty acid synthase; *acox-3:* Acyl-CoA Oxidase 3; *agpat4:* 1-Acylglycerol-3-Phosphate O-Acyltransferase 4; *dgat2:* Diacylglycerol O-Acyltransferase 2; *hnf4α:* hepatocyte nuclear factor 4α; *lepr*: leptin receptor; *npy*: neuropeptide Y; *mc4r:* melanocortin 4 receptor.

Tables 2 & 3. **Levels of Endocannabinoids**. Levels Anandamide (AEA); 2-arachidonoylglycerol (2-AG); Palmitoylethanolamide (PEA) and Oleoylethanolamine (OEA) in the brain (table 1) and liver (table 2) of female zebrafish treated with different doses of DiNP. AEA measured as pmol/gr tissues; 2-AG, PEA and OEA as pmol/mg tissues. DiNP doses comparted with CONTROL group. CTRL: control. (*) indicate significant differences evaluated by one-way ANOVA with Tukey's post – test ($p < 0.05$). Levels expressed as mean of 5 animals ± SD.

Figure 1. ECS gene expression in the brain of zebrafish exposed to three different doses of DiNP. qPCR analysis of genes encoding for ECS receptors (A), enzymes for AEA and 2-AG metabolism (B). Each single point consists of five female brain expressions (relative expression ± SD as determined by Bio-Rad Laboratories iQ5 manager software). CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test).

Figure 2. ECS gene expression for appetite signals in brain (*npy, mc4r, lepr*). Each single point consists of five female brain expressions (relative expression ± SD as determined by Bio-Rad Laboratories iQ5 manager software). CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test).

Figure 3. ECS gene expression in the liver of zebrafish exposed to three different doses of DiNP. qPCR analysis genes encoding ECS receptors (A), enzymes for AEA and 2-AG metabolism (B). Each single point consists of five female liver expressions (relative expression ± SD as determined by Bio-Rad Laboratories iQ5 manager software). CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test).

Figure 4. qPCR analysis for genes encoding lipid metabolism signals in liver of zebrafish exposed to three different doses of DiNP. Each single point consists of five female liver expressions (relative expression ± SD as determined by Bio-Rad Laboratories iQ5 manager software). CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test).

Figure 5. Histological slices of liver and percentage of liver covered by lipid vacuoles. Panel with representative pictures of female zebrafish liver exposed to three-week treatment of DiNP; Control (a); 0.42 µg/L (b); 4.2 µg/L (c); 42 µg/L (d). Scale bar: 3 µM. (A). Graphic showing the results from the ImageJ analysis. Each point consists in the analysis of 5 pictures randomly taken in each liver, with a total of 7 livers per experimental group and expressed in percentage respect the total area of the hepatic tissue. (mean ± SD, $p < 0.05$). CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test) (B).

Figure 6. FAAH enzymatic activity in liver membrane cells measured as pmol / min / mg of protein. CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test). Data expressed in mean ± SD.

Figure 7. Chemical maps integrated under the lipids stretching regions (2990-2836 cm^{-1}). Panel of pictures with the chemical analysis of lipids in liver. Control (a); 0.42 µg/L (b); 4.2 µg/L (c); 42 µg/L (d). The color represents the total amount of lipid in the tissue. False color scale on the right (A). Graphics showing the results from FT-IR analysis. Each point consists of the analysis of 2 regions randomly taken in each liver, with a total of 3 livers per experimental group (mean ± SD). Graphics are expressed in a.u. (arbitrary units). CTRL: control. Letters above each column indicate statistical differences among groups ($p < 0.05$ vs. untreated controls; ANOVA followed by Tukey's multiple comparison test).

Figure 8. Summarized findings. Abbreviations used: DiNP: di-isononyl phthalate; ECS: endocannabinoid system; EC: endocannabinoids; EC-like: endocannabinoid – like mediators.

Table 1.

Primer	Forward (5'-3')	Reverse (5'-3')	Ta (ºC)	Accession number
arp	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	60	NM131580.2
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	59	NM_212784.1
cnr1	TCTGTGGGAAGCCTGTTTC	ACCGAGTTGAGCCGTTTG	55	NM_212820.1
cnr2	CCATCGTACCTGTTCATTAG	GCTGTCAGCAAAAGGCTTC	58	BC163057.1
daglα	GAGGGTTTCCGTCGTCAC	TGTTCCTCCAGCAATGATCC	59	XM_692781
mgll	AAGTGAAGGTGAGAGGAT	AATGTCCAACGATGAAGA	53	NM_200297
nape-pld	CTCAAGGACATGCACTCA	GAGCACAATCTTCAAGACAAT	57	NM_001080613
abhd4	GAAGAGCAGTTTGTTTCCTCCATAG	GACTCACTCTTTCTGGGTATTGGAT	60	NM_001017613.1
faah	CATTCTCGGACCAGCCTTCA	CCTTCACTGCCTGTTGGAAGA	60	NM_001109825.1
cox-2	ATCCAGGATGAAGTCTACAAT	GCTGTTGACGCCATAATC	57	NM_001025504
pparα	TCCACATGAACAAAGCCAAA	AGCGTACTGGCAGAAAAGGA	60	NM_001161333
pparγ	CTGCCGCATACACAAGAAGA	TCACGTCACTGGAGAACTCG	60	NM_131467.1
fasn	ATGAAACACACAGGGACTCAGG	TTCTTGAATCTGAACGCGGGTA	60	XM_009306806.2
acox3	AAGGACATCGAGCGAATGAT	CTATGAAAGAGTGGAGGCCG	59	NM_213147.1
agpat4	TGCTGAAAACTCAGTTGCTG	AGTAACCCAGTCTGCAGTTG	56	NM_212992.1
dgat2	TTCCGGTGTCAAAAAGGGCT	CAGCAGCAAAGAGCAAGCAA	58	NM_001030196.1
hnf4a	ACGGTTCGGCGAGCTGCTTC	TCCTGGACCAGATGGGGGTGT	58	NM_194368.1
lepr	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	59	NM_001309403.1
npy	GTCTGCTTGGGGACTCTCAC	CGGGACTCTGTTTCACCAAT	59	NM_131074.2
mc4r	CCCGCGGAATCCTTATTGTG	CCAGTTGCTAAGTGCTGTCA	60	NM_173278.1

Table 2.

	AEA (pmol/g)	2-AG (pmol/mg)	PEA(pmol/mg)	OEA(pmol/mg)
CONTROL	18.30 ± 3.38	2.92 ± 0.81	0.29 ± 0.08	0.24 ± 0.09
0.42µg / L	15.05 ± 4.94	3.24 ± 0.44	0.26 ± 0.08	0.24 ± 0.05
4.2µg / L	6.80 ± 1.94**	2.54 ± 1.05	0.17 ± 0.04	0.17 ± 0.09
42µg / L	8.60 ± 4.32*	3.48 ± 0.41	0.21 ± 0.07	0.09 ± 0.08*

Table 3.

	AEA (pmol/g)	2-AG (pmol/mg)	PEA(pmol/mg)	OEA(pmol/mg)
CONTROL	4.80 ± 1.19	4.92 ± 2.63	0.05 ± 0.03	0.13 ± 0.05
0.42µg / L	5.87 ± 1.50	4.92 ± 2.56	0.21 ± 0.07*	0.03 ± 0.03**
4.2µg / L	10.48 ± 2.29***	2.50 ± 1.60	0.19 ± 0.09*	0.05 ± 0.00*
42µg / L	8.00 ± 1.64*	6.74 ± 1,90	0.09 ± 0.07	0.10 ± 0.04

Figure 1a.

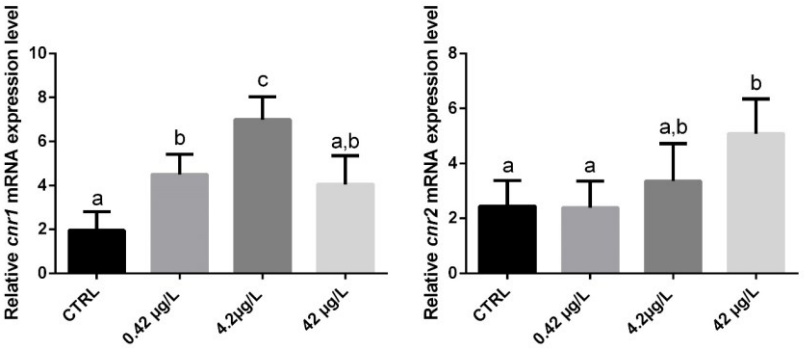


Figure 1b.

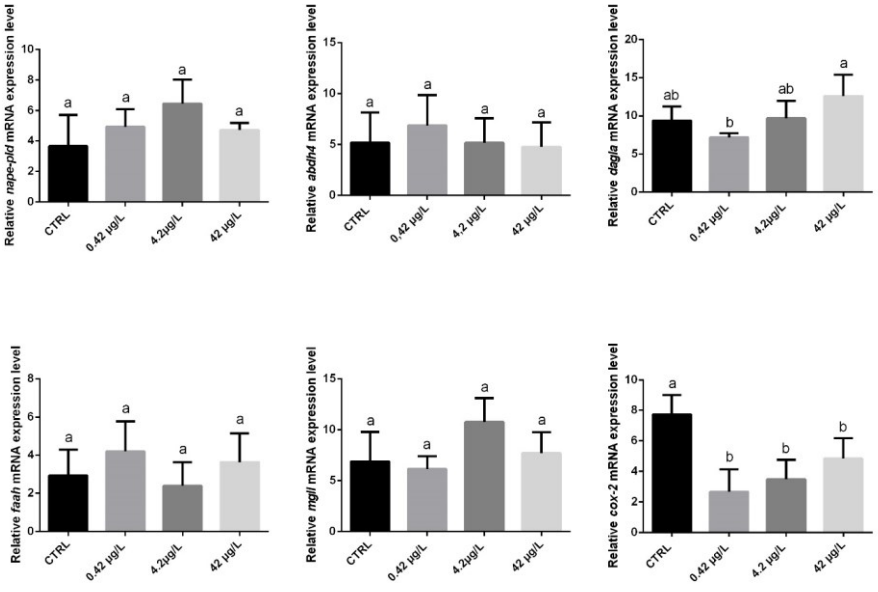


Figure 2

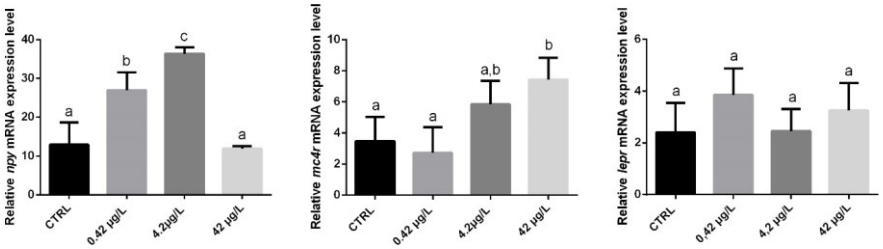


Figure 3a

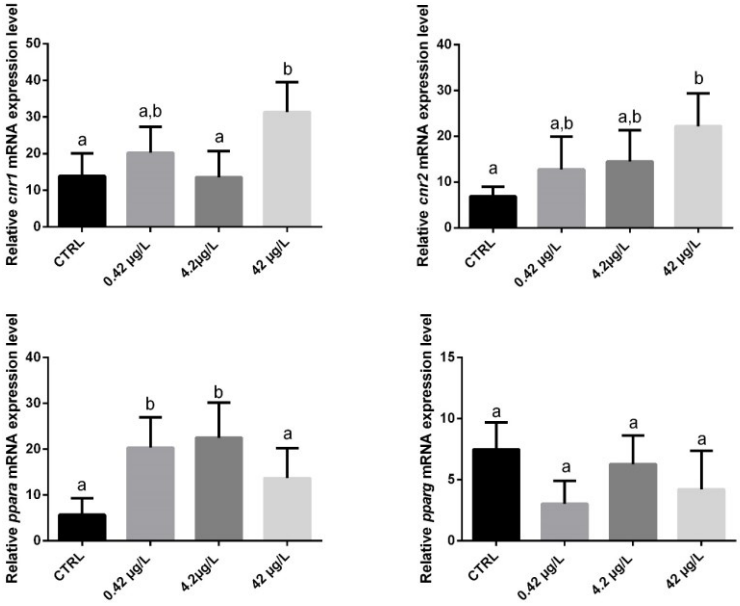


Figure 3b.

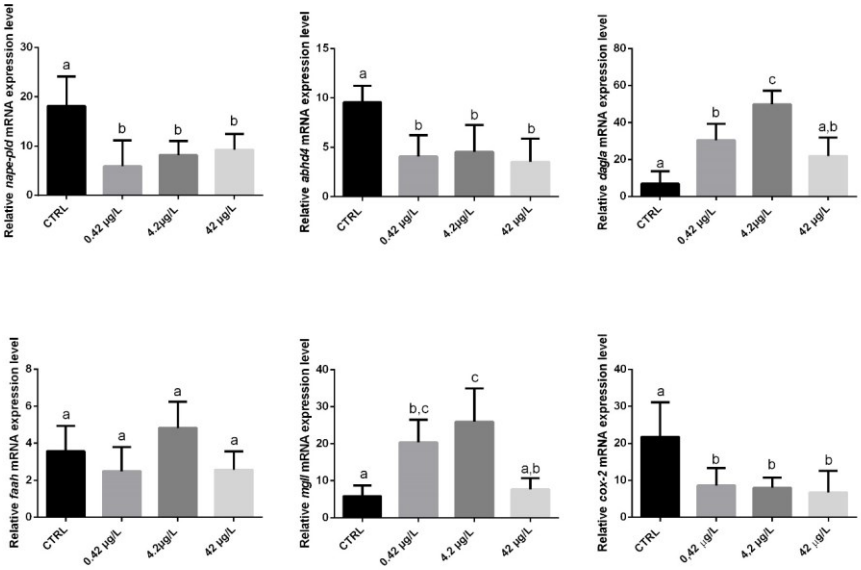
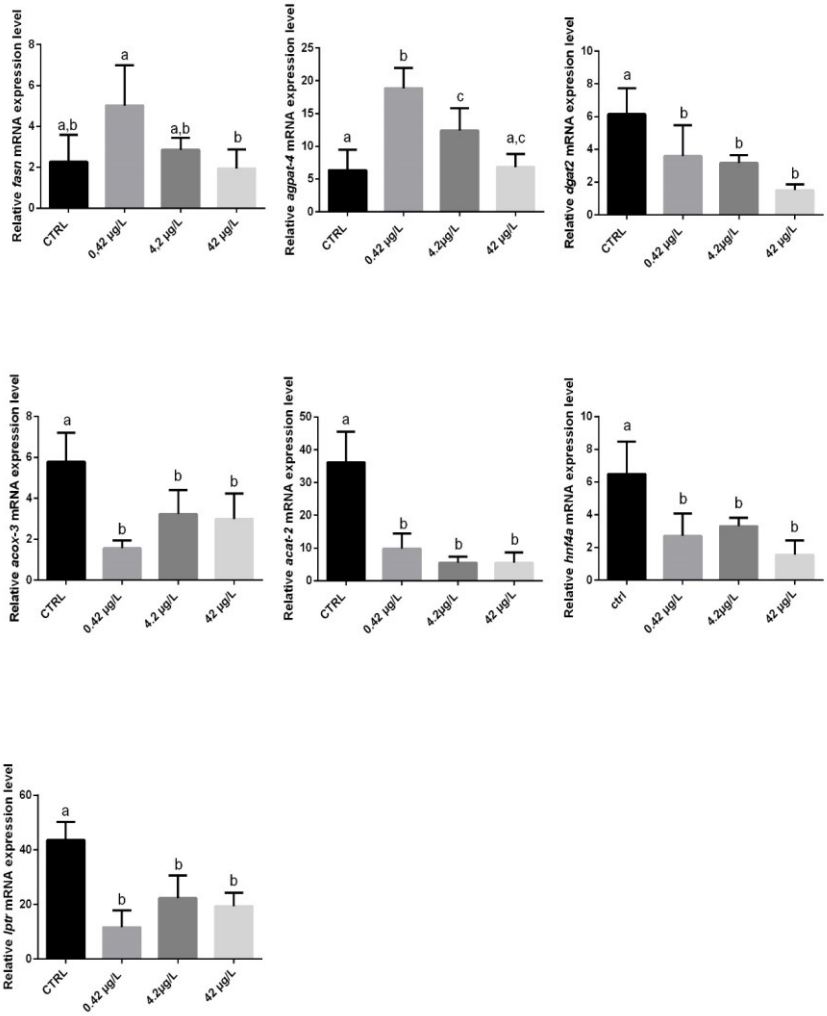


Figure 4


Relative fasn mRNA expression level
a,b
a
a,b
b
CTRL
0.42 µg/L
4.2 µg/L
42 µg/L
Relative agpat-4 mRNA expression level
a
b
c
a,c
Relative dgat2 mRNA expression level
a
b
b
b
Relative acox-3 mRNA expression level
a
b
b
b
Relative acat-2 mRNA expression level
a
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ctrl
Relative lptr mRNA expression level
a
b
b
b

Figure 5a.

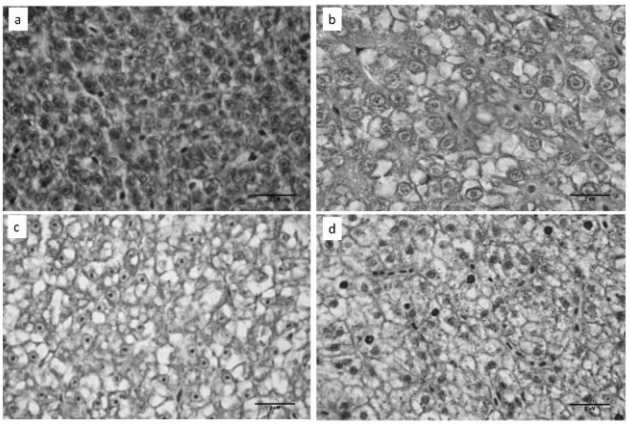

Figure 5b.

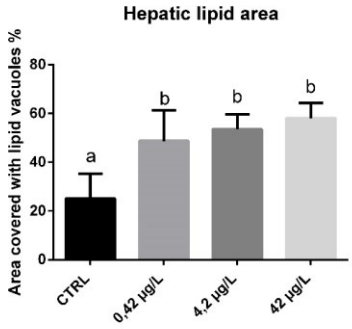

Figure 6.

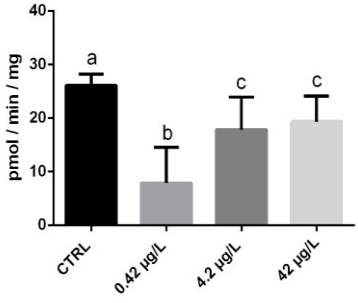

Figure 7a

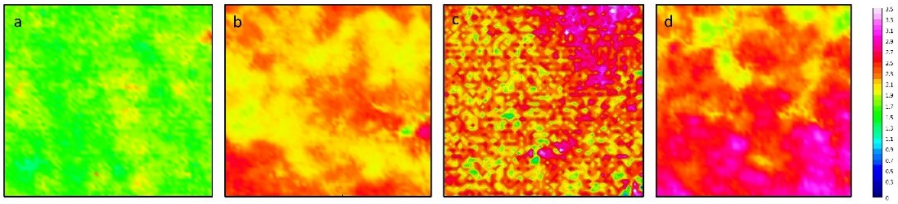

Figure 7b.

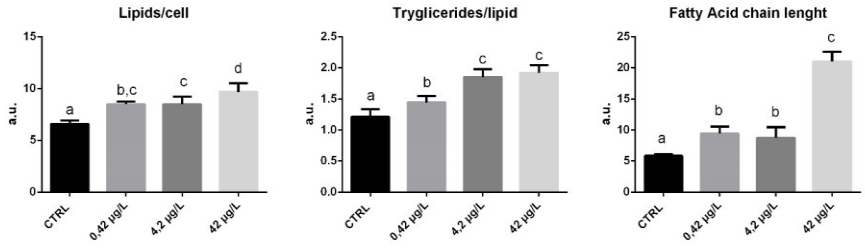

Figure 8.

DOSE-EFFECT ALTERATIONS INDUCED BY DiNP	0.42 µg/L	4.2 µg/L	42 µg/L
Central ECS	✓	✓	✓
Central EC levels		✓	✓
Central EC-like levels			✓
Food intake markers	✓	✓	✓
Peripheral ECS	✓	✓	✓
Peripheral EC levels		✓	✓
Peripheral EC-like levels	✓	✓	
Lipogenic and lipolytic markers	✓	✓	✓
Fatty liver	✓	✓	✓

CHAPTER 2

Effects of Bisphenol A on the liver and brain of zebrafish

Role of Bisphenol A on the Endocannabinoid System at central and peripheral levels: effects on adult female zebrafish

Forner-Piquer *et al*.

- Manuscript in preparation -

Abstract

Bisphenol A (BPA), a widely used plasticizer, has become a ubiquitous pollutant due to its extensive use. Its endocrine disrupting properties have been documented in several studies, as well as its potential to induce metabolic and reproductive impairments at environmentally relevant concentrations. Recent insights highlighted the role of the Endocannabinoid System (ECS) in energy homeostasis and lipid metabolism. Disruption of the ECS, may induce metabolic alterations among other effects. The main objective of this study was to investigate the disruptive effects of environmentally relevant concentrations of BPA on the ECS in female zebrafish liver and central nervous system. Adult female zebrafish were exposed for 3 weeks to three different concentrations of BPA (5 µg/L; 10 µg/L; 20 µg/L). We observed changes in the expression of a number of genes involved in the Anandamide (AEA) and 2-Arachidonoylglycerol (2-AG) metabolism in the liver and brain, as well as altered levels of endocannabinoids and endocannabinoid-like mediators. These changes were associated with greater presence of hepatic lipid vacuoles, following exposure to the highest concentration of BPA (20 µg/L) tested, although there were no changes in food intake and in the expression of the molecular markers for appetite. The overall results support the hypothesis that exposure to BPA induced changes in brain and liver ECS system of adult female zebrafish causing the increase of hepatic fat at the highest concentration tested, but not via food intake.

Highlights:

a- BPA altered the ECS signaling in adult female zebrafish.
b- 20 µg/L BPA increased the presence of fat in the liver.
c- BPA did not modify the food intake.

Keywords: BPA, liver, endocannabinoid, zebrafish, appetite.

Abbreviations: BPA: bisphenol A, ECS: Endocannabinoid System, EC: endocannabinoids, EDC: endocrine disruptor chemical. AEA: anandamide, 2-AG: 2-arachidonoylglycerol, PEA: N-palmitoyl-ethanolamide, OEA: N-oleoyl-ethanolamide, CB1/cnr1: endocannabinoid receptor type I, CB2/cnr2: endocannabinoid receptor type II, TRPV1: transient receptor potential cation channel subfamily V member 1, GPR55: G coupled protein 55, NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D, FAAH: *Fatty acid amide hydrolase, DAGLα:* Diacylglycerol Lipase alpha*, MGLL:* Monoacylglycerol lipase*, COX-2: cyclooxigenase-2, ABDH4:* α/β-hydrolase 4, NPY: neuropeptide Y, LEPR: leptin receptor, MC4R: melanocortin 4 receptor.

Declarations of interest: none.

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Introduction

Bisphenol A (BPA) is one of the most widely used plasticizers and its annual manufacture was predicted to surpass 5.4 million tons by 2015 (Merchant Research & Consulting, 2014). BPA, which is used mainly in the production of epoxy resins

and polycarbonates (Staples et al., 1998), has become ubiquitous in the environment due to its presence in a large range of consumable and daily products. According to a number of studies, BPA can be found in all environmental matrices, including water, soil and air (Corrales et al., 2015). In the aquatic environment, BPA contamination can be traced back to wastewater discharges and landfill sites (Kang et al., 2007) with varying concentrations depending on the location and depth of the sampling place (Flint et al., 2012) which can reach to a high level of 56 µg/L in surface waters (Corrales et al., 2015). Anyhow, for BPA, 12 µg/L or lower, are considered as environmental relevant concentrations (Flint et al., 2012). Even BPA is a fat-soluble and tends to accumulate in fatty tissues, studies suggested that it is rapidly metabolized in teleost (Bjerregaard et al., 2007; Lindholst et al., 2001) and has a relatively short half-life (Staples et al., 1998). In this context, BPA has been detected in tissues of several aquatic species. (Corrales et al., 2015; Mita et al., 2011).

Several studies have demonstrated that BPA can exert estrogen-like activity as well as interacting with androgen receptors (Kinch et al., 2015), resulting in the impairment of reproduction and development by disrupting endocrine functions (Canesi and Fabbri, 2015; Hatef et al., 2012; Oehlmann et al., 2009; Vom Saal and Hughes, 2005). More recently, BPA was shown to have obesogenic effects (Pereira-Fernandes et al., 2013). In this line, in mouse exposed to five different concentrations of BPA via diet (0, 5, 50, 500, and 5000 µg/kg/day), the lower doses upregulated the expression of lipogenic genes and contribute to hepatosteatosis (Marmugi et al., 2012). BPA was also reported to increase liver weight and fat mass in male offspring from pregnant mice fed with BPA (Angle et al., 2013). Increased lipid content was also reported in HepG2 human hepatoma cells treated *in vitro* (Huc et al., 2012), and in mice exposed to 0.5 µg BPA /kg/day, was suggested that BPA may induce hepatic lipid accumulation via epigenetic reprogramming of genes involved in lipid metabolism (Ke et al., 2016). In sea bream, dietary exposure to BPA

(Maradonna et al., 2015, 2014), resulted in alterations of the hepatic lipid metabolism and accumulation of lipids vacuoles. At central level, exposure to BPA also induced alterations of brain development, structure and behavior (Itoh et al., 2012; Richter et al., 2007; Wolstenholme et al., 2011) in both mice and zebrafish (Kinch et al., 2015; Saili et al., 2012). Furthermore, exposure to low concentration of BPA (0.0068 µM) was shown to induce abnormal hypothalamic cell turnover and increase of hyperactivity in developing zebrafish (Kinch et al., 2015).

Exposure of zebrafish to 100µg/L BPA for 48h was demonstrated to induce accumulation of lipid vacuoles in the liver via endocannabinoid-mediated positive feedback loop (Martella et al., 2016). Specifically, the Endocannabinoid System (ECS) is a lipid signaling system involved in a number of physiological functions, which may be affected by endocrine disruptive chemicals (EDCs) (Forner-Piquer et al., 2017). There is evidence that the ECS is involved in the modulation of energy balance (Di Marzo and Matias, 2005), food intake and lipid metabolism where a correct endocannabinoid tone is essential for the well-being of the organisms. Briefly, the two endocannabinoids (EC), Anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are lipids derived from cell membrane phospholipids, acting via cannabinoid G-protein coupled receptors of type 1 and 2 (CB1 and CB2). The ECS includes endocannabinoids and their receptors, as well as the enzymes that regulate the production and degradation of AEA and 2-AG. The best studied of these are N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and Fatty acid amide hydrolase (FAAH) for the biosynthesis and catabolism of AEA respectively as well as Monoglyceride lipase (MGLL) and Diacylglycerol Lipase Alpha (DAGLα) for 2-AG. In the liver, the ECS is involved in the control of hepatic function and lipid metabolism. Indeed, the upregulated expression of *cb1* and/or *cb2* (Deveaux et al., 2009; Purohit et al., 2010) and increased levels of AEA and/or 2-AG are found in steatotic conditions (Martella et al., 2016; Osei-Hyiaman et al., 2008, 2005). At central level, the ECS is involved in the stimulation of appetite, for

instance, in teleosts, administration of AEA was shown to increase food intake (Piccinetti et al., 2010b; Valenti et al., 2005).

The aim of the present study was to investigate the adverse effects of chronic exposure to environmentally relevant concentrations of BPA in adult female zebrafish. Emphasis is placed on the effects of BPA on the ECS in the liver and the brain, as well as the possible disruption of food intake and the hepatic lipid accumulation after 21-day treatment since effects on the ECS has been exclusively reported after acute administration of BPA for 48h (100µg/L) in zebrafish (Martella et al., 2016).

Methods

Animals and BPA exposure. The experiments, in duplicate, were carried out using one-year old female, sexually mature zebrafish (*Danio rerio*, wild-type strain) in 100-L aquaria with recirculating system following Forner-Piquer et al., (2017). Three nominal concentrations of BPA (Sigma-Aldrich, Oakville, Canada) were used diluted in 1mL of 100% ethanol (vehicle): 5 µg/L, 10 µg/L, 20 µg/L and added to the water. Since the ethanol used as BPA solvent did not exceed 100µL/L in the tank, the control group was reared only in fresh water without ethanol, according to Organization for Economic Cooperation Development for chronic assays (OECD 2012)

Twenty female fishes were distributed in each tank and chronically exposed for 21 days. The BPA concentrations used were selected on the base of previous published papers (Crain et al., 2007; Flint et al., 2012; Santangeli et al., 2016). After twenty-one days of treatment, fish were euthanized with buffered MS-222 (3-aminobenzoic acid ethyl ester) (Sigma-Aldrich, Oakville, Canada) according to University of Calgary Animal Care protocol (# AC15-0183), and sampled at 8-10 a.m. Livers and brains from each experimental group were extracted, frozen on dry ice

and stored at -80ºC for RNA extraction and endocannabinoids assays. In addition, livers were fixed in Bouin solution for histology. Given the smaller size of the fish, each fish was considered as a biological replicate.

Food intake analysis. Food intake (FI) was measured according to (Piccinetti et al., 2010a) at the end of the experiment giving a pre-weighted amount of food to the fishes and collecting the non-ingested pellets after 5 hours from administration.

RNA extraction and cDNA synthesis. Total RNA was isolated from the liver and brain with RNAzol solution (Sigma Aldrich, Milan, Italy) according to the manufacturer's instructions and methods described by Santangeli et al., (2016). Final RNA concentrations were determined with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Monza, Italy) while integrity was verified by gel red staining of 28S and 18S ribosomal RNA bands on a 1% agarose gel. Three micrograms of total RNA was used for cDNA synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy) and kept at −20 °C until use.

Real-Time qPCR. The relative quantification of gene expressions was performed with the SYBR green method in an iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad, Milano, Italy) following Santangeli et al., (2016).

The internal controls used included *18s* and *rplp0* for liver, and *rplp0* and *b2m* for brain (McCurley and Callard, 2008; Roesner et al., 2006; Tang et al., 2007). Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad) including Genex Macro iQ5 Conversion and Genex Macro iQ5 files. The calculations of the mRNA expression levels were derived from the algorithms outlined by Vandesompele et al., (2002) and from the geNorm manual. Data were generated in duplicated and expressed as relative mRNA levels (Arbitrary Units). Primers used at a final concentration of 10 pmol/µL (Table 1).

Measurement of endocannabinoids (AEA, 2-AG) and endocannabinoid-like mediators (PEA, OEA) in the liver and brain. Endocannabinoids and endocannabinoid-like mediators were extracted, purificated and quantificated from liver and brain as described previously by Di Marzo et al., (2001).

Liver Morphology. Livers from BPA treatments and control groups were fixed in Bouin and prepared for histological examination following Forner-Piquer et al., (2017).

Area covered with lipids vacuoles in the liver (ACVL%). The estimation of hepatic lipid area was done according to the methodology of Papadakis et al., (2013). For each liver sample, five microphotographs were obtained at ×100 magnification from different areas of the liver. Then, the photographs were analyzed with ImageJ (ImageJ, NIH, USA).

Statistical analysis. When data fulfilled the conditions to apply a parametric test, one-way ANOVA followed by Tukey's multiple comparison test was used, when not, Kruskal-Wallis (non-parametric test) was applied. Confidence interval was set at 95% ($p < 0.05$). Results are presented as mean ± standard deviation (SD). All statistical procedures were performed using *GraphPad Prism 6* and *SigmaStat 3.5.* Letters above each column indicate statistical differences among the treatments.

Results

Food intake. The amount of food intake was not affected following exposure to BPA (Figure 1a). In addition, genes coding for three key signals for the appetite at central level were analyzed. Accordingly, there were no changes in the transcript levels for *npy*, (Figure 1b), leptin receptor *lepr* (Figure 1c) and *mc4r* (Figure 1d).

Gene expression in liver and brain. To test the effects of BPA exposure on the ECS pathway, we measured the transcript levels for ECS genes in the central and peripheral levels.

In the brain, while no changes were observed for the endocannabinoid receptor type I *(cnr1)* (Figure 1a), BPA reduced the transcript levels for genes coding for the endocannabinoid receptor type II (*cnr2*), G protein coupled–receptor 55 (*gpr55*) and the transient receptor potential cation channel subfamily V member 1 (*trpv1)* (Figure 2b, 2c, 2d). Regarding genes coding for enzymes involved in the endocannabinoid pathway, exposure to BPA did not alter the transcriptional levels of *nape-pld* (Figure 3a), the enzyme mediating the biosynthesis of AEA. On the contrary, BPA exposure at the highest concentration increased transcript level for the gene encoding for *faah* (Figure 3b), which is an enzyme involved in the hydrolysis of AEA. Also involved in the metabolism of AEA and 2-AG into oxidation products is the cyclooxygenase-2 (*cox-2*) (figure 3c) which mRNA levels were decreased after BPA exposure. In this line, the treatment was without effect on the expression of the gene coding for *dagla,* enzyme responsible for the metabolism of 2-AG (figure 3d), however the lowest concentration reduced *mgll* transcripts (Figure 3e), which is a 2-AG catabolic enzyme.

In the liver, BPA was without effect on the mRNA levels of the receptors *cnr1*, *trpv1* and *gpr55* (5a, 5c, 5d) excepting for *cnr2* (figure 5b). Regarding the genes coding for the enzymes involved in the biosynthesis of endocannabinoids, the levels of *nape-pld* (Figure 6a), *abdh4* (Figure 6b) and *dalgα* (Figure 6c) were increased at 5 µg/L group, excepting *abdh4,* which was increased at 5 and at 20 µg/L. For the genes coding for the catabolic enzymes, *faah* (figure 6d) was unchanged, different from *cox-2* (Figure 6d) which was reduced after all the BPA treatments. Finally, *mgll* (figure 5f) was not affected.

Endocannabinoids and Endocannabinoid-like mediator's levels in liver and in brain. Continuing with the study of ECS, in the brain, the exposure to BPA increased the levels of AEA (Figure 6a), but reduced the 2-AG at 10 µg/L (Figure 6b). About the EC-like mediators, BPA induced the reduction of PEA only significantly at 5 µg/L (Figure 6c), without effect on OEA (Figure 6d).

In the liver**,** BPA exposure increased the levels of AEA (Figure 7a) but not 2-AG (Figure 7b). Regarding the EC-like mediators, PEA was unchanged after the treatments while OEA was altered only at the medium concentration (Figure 7d).

Hepatic histology in BPA treatment. We investigated the potential incidence of hepatosteatosis associated with changes in central and peripheral ECS following a BPA treatment. The 20 µg/L of BPA increased the size of the hepatic lipid vacuoles, as shown in the histological panel (Figure 8a). In accordance with the hepatic morphology, a significant increment of the ACVL in fishes exposed to the highest BPA concentration (20 µg/L) was measured (Figure 8b). 5 and 10 µg/L BPA groups were without effect on the ACVL.

Discussion

A number of investigators suggested a connection among sex steroids (*i.e*. estradiol) and the ECS. For instance, estradiol (E2) has been reported to upregulated *faah* mRNA levels and FAAH activity in mouse Sertoli cells (Grimaldi et al., 2012; Rossi et al., 2007). In this line, the analysis of the proximal 5' flanking region of the *faah* gene showed the presence of three potential estrogen receptor-binding sites (Grimaldi et al., 2012; Waleh et al., 2002). Consequently, the levels of AEA fluctuated with E2 and furthermore, the expression of *cb1* in rats (González et al., 2000).

Thus, bearing in mind the estrogen-like activity of BPA, the main goal of this study was to evaluate whether chronic exposure to environmental relevant concentrations of BPA might induce alterations in the ECS signaling.

Martella and collaborators (2016) were the first to demonstrate BPA-induced fatty liver in zebrafish via disruption of ECS both at central and peripheral level after acute exposure of adult zebrafish to 100 µg/L BPA (48 hours). The results herein reported are consistent with the aforementioned study, where BPA increased the

levels of hepatic AEA concomitant with the increased expression of the genes coding for the AEA biosynthetic enzymes (*nape-pld* and *abdh4*) and a reduction on the transcript levels of *cox-2*, enzyme responsible for the deactivation of AEA. As previously shown, fatty liver typically display an elevated level of AEA and greater expression of *cnr1* in hepatocytes which underlies endocannabinoid-mediated triglyceride biosynthesis (Osei-Hyiaman et al., 2008, 2005, Martella et al 2016). While the levels of AEA were raised in all treated groups, the mRNA levels of *cnr1* remained unchanged. In addition, the other main receptor, *cnr2*, which has been associated with fatty liver and potentiation of obesity in mice (Deveaux et al., 2009) and humans (Mendez-Sanchez et al., 2007), was induced only by the 5 µg/L BPA group. It seems that the increase of fat may be not mediated via *cnr1/cnr2* in this case but via other lipogenic pathways.

Regarding the EC-like mediators, OEA, is produced and inactivated thought similar pathways to those of AEA but does not bind cannabinoids receptors (Fu et al., 2003). It was shown that OEA mitigates steatosis in liver (Li et al., 2015) and accordingly, its level was found to be higher in fishes exposed to 10 µg/L of BPA, linked with the liver showing the lowest lipid area. Concerning PEA, to date, no studies are available relating its possible role in hepatosteatosis process.

Moving to the central ECs, deeply involved in the control of appetite, since CB1 is considered a key factor in the neural control of food intake, which is stimulated by exogenous and endogenous cannabinoids agonist. For instance, central CB1 antagonist was found to decrease food intake in rats (Colombo et al., 1998; Freedland et al., 2000) and obese mice (Di Marzo et al., 2001), while administration of anandamide induces stimulation of the appetite via CB1 (Williams and Kirkham, 1999; Jamshidi and Taylor, 2001). It should be noted that contradictory results have been reported concerning the ECS in central nervous system when animals were chronically exposed to BPA. In rats exposed to 10 µg/mL BPA via water during perinatal period, BPA was found to exert an anorexigenic effect by reducing

hypothalamic *cnr1* expression (Suglia et al., 2016). By contrast, Martella et al., (2016) found an increase of the *cnr1* expression in zebrafish following acute (48h) exposure to 100 µg/L BPA. In the present study, chronic exposure to lower concentrations of BPA did not alter *cnr1* transcript abundance, revealing the complexity of BPA activity possibly due to non-monotonic action of BPA. Regarding CB2, there are a number of opposite observations with respect to the role of CB2 in the regulation of food intake. Emadi and collaborators (2011) demonstrated that treatment with a CB2 agonist, CB65, induces food intake in neonatal chicks. In accord with this observation, treatment with a CB2 antagonist, AM630, inhibits food consumption in certain strains of mice (Onaivi et al., 2008). But Agudo et al. (2010) reported that deficiency of CB2 cannabinoid receptor in mice enhance food intake and obesity with age and Ting and coworkers (Ting et al., 2015) that administration in rats of AM630, also increased food intake. Anyhow, the biological significance of the downregulation of *cnr2* induced by BPA does not seem to alter the food intake in zebrafish, as demonstrated with the measurement of food intake *in vivo* and the markers for the appetite (*npy, lepr*, *mc4r*). As for the GPR55 receptor, latest findings indicate that GPR55 may play a role in regulation of metabolism (Henstridge et al., 2016). In humans GPR55 is positively associated with obesity, while in mice, GPR55 deletion was found to increase adiposity by decreasing physical activity (Meadows et al., 2016). Thus, similarly to CB2, GPR55 role seems to be not fully established, as the possible role of TRPV1 in appetite.

Conclusions

Summarizing, it is likely that the observed increase of the lipid vacuoles content in the hepatic tissue at the highest BPA concentration may be due to changes in the lipogenesis and lipolysis pathways, jointly with a deregulation of the levels of EC and EC-like mediators, rather than changes in energy intake. The findings here reported provided the ECS as a possible new target for xenobiotics exposure and a

contribution to the BPA Adverse Outcome Pathway (Figure 9) for the toxicity assessment of BPA on freshwater organisms.

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Figures & tables

Table 1. *18s*: ribosomal subunit 18s; *rpp0* ribosomal protein large p0; *b2m*: beta -2-microglobulin; *cnr1*: endocannabinoid receptor type I; *cnr2*: endocannabinoid receptor type II; *trpv1*: transient receptor potential cation channel subfamily V member 1; *gpr55*: G coupled protein 55; *nape-pld*: N-acyl phosphatidylethanolamine phospholipase D; *faah*: *Fatty acid amide hydrolase; daglα:* Diacylglycerol Lipase alpha*; mgll:* Monoglyceride lipase*; cox-2/ptgs2b: cyclooxigenase-2 / prostaglandin-endoperoxide synthase 2b; abdh4:* α/β-hydrolase 4*; npy: neuropeptide Y; lepr: leptin receptor; mc4r: melanocortin 4 receptor.*

Figure 1. Appetite. Food intake measured *in vivo* as food intake per mg/g of Body Weight (a). Transcriptional profiles of genes coding for appetite markers in the brain, neuropeptide Y (b), leptin receptor (c) and melanocortin 4 receptor (d); data expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5 manager software. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

Figure 2. Transcriptional profiles of genes coding for endocannabinoid receptors in the brain: endocannabinoid receptor type I (a), endocannabinoid receptor type II (b), G coupled receptor 55 (c), transient receptor potential cation channel subfamily V member 1 (d); expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5 manager software. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

Figure 3. Transcriptional profiles of genes coding for endocannabinoid metabolic enzymes in the brain: N-acyl phosphatidylethanolamine phospholipase D (a), *fatty acid amide hydrolase (b); cyclooxigenase-2 / prostaglandin-endoperoxide synthase 2b (c), di*acylglycerol lipase alpha (d)*,* monoglyceride lipase (3e); expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5

manager software. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

Figure 4. Transcriptional profiles of genes coding for endocannabinoid receptors in the liver: endocannabinoid receptor type I (a), endocannabinoid receptor type II (b), G coupled receptor 55 (c), transient receptor potential cation channel subfamily V member 1 (d); data expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5 manager software. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

Figure 5. Transcriptional profiles of genes coding for endocannabinoid metabolic enzymes in the liver: N-acyl phosphatidylethanolamine phospholipase D (a), α/β-hydrolase 4 (b), *fatty acid amide hydrolase (c); cyclooxigenase-2 / prostaglandin-endoperoxide synthase 2b (d), di*acylglycerol lipase alpha (e), monoglyceride lipase (f); expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5 manager software. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

Figure 6. Endocannabinoids levels in the brain: anandamide (a), 2-arachidonoylglycerol (b) and endocannabinoid-like mediators: palmitoylethanolamide (c), oleoylethanolamine (d). Data are expressed as pmol/mg ± SD except for AEA which is reported as: pmol/g ± SD. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

Figure 7. Endocannabinoids levels in the liver: anandamide (a), 2-arachidonoylglycerol (b) and endocannabinoid-like mediators: palmitoylethanolamide (c), oleoylethanolamine (d). Data are expressed as pmol/mg ± SD except for AEA which is reported as: pmol/g ± SD. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$).

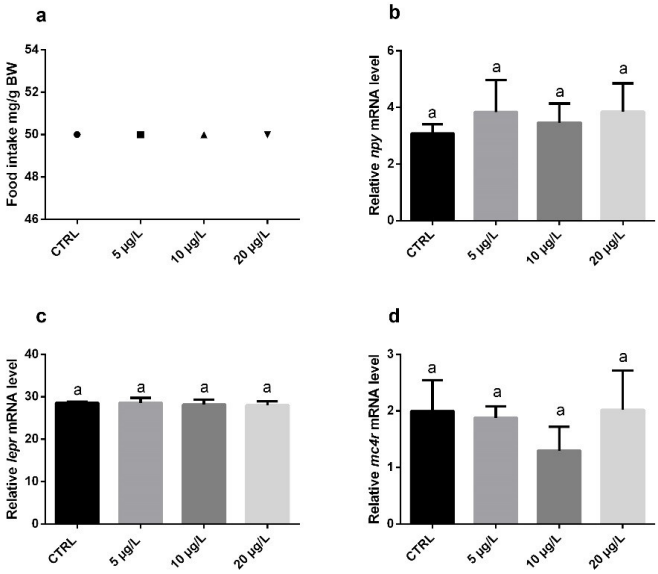
Figure 8. Mayer's hematoxylin – eosin histology of zebrafish liver treated with BPA (A). Control group (a); 5 µg/L BPA (b); 10 µg/L BPA (c); 20 µg/L BPA (d). Scale bar: 2µm. Histogram of the percentage of hepatic tissue covered by lipids vacuoles (ACLV) of livers from fishes exposed to BPA. Data expressed in percentage as means ± SD. CTRL: control. Letters above each column indicate statistical differences among groups (one-way ANOVA, $p < 0.05$) (B).

Figure 9. Adverse Outcome Pathway for BPA in female zebrafish treated with environmental concentrations of BPA based on altered ECS and the increased hepatic fat at the highest BPA concentration.

Table 1.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Acc number	Ta (ºC)
18s	TCGAATGTCTGCCCTATCAACT	AGACTTGCCCTCCAATGGATC	AF308735	55
rplp0	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM_131580.2	60
b2m	GCCTTCACCCCAGAGAAAGG	GCGGTTGGGATTTACATGTTG	L05383.1	60
cnr1	TCTGTGGGAAGCCTGTTTC	ACCGAGTTGAGCCGTTTG	NM_212820.1	56
cnr2	CCATCGTACCTGTTCATTAG	GCTGTCAGCAAAAGGCTTC	BC163057.1	59
trpv1	TGATCGTCGCTGGTGCTT	GACTGGGCTCTCTCTGAACG	KX096869.1	59
gpr55	AACTGAAGGTGTGGATAC	ACGCCATAATGTTTAACG	XM_005163567.2	53
nape-pld	CTCAAGGACATGCACTCA	GAGCACAATCTTCAAGACAAT	NM_001080613	57
faah	CATTCTCGGACCAGCCTTCA	CCTTCACTGCCTGTTGGAAGA	NM_001109825.1	60
daglα	GAGGGTTTCCGTCGTCAC	TGTTCCTCCAGCAATGATCC	XM_692781	59
mgll	AAGTGAAGGTGAGAGGAT	AATGTCCAACGATGAAGA	NM_200297	53
cox-2/ptgs2b	ATCCAGGATGAAGTCTACAAT	GCTGTTGACGCCATAATC	NM_001025504	57
abdh4	GAAGAGCAGTTTGTTTCCTCCATAG	GACTCACTCTTTCTGGGTATTGGAT	NM_001017613.1	60
npy	GTCTGCTTGGGGACTCTCAC	CGGGACTCTGTTTCACCAAT	NM_131074.2	59
lepr	CTCCAGTGACGAAGGCAACTT	GGGAAGGAGCCGGAAATGT	NM_001309403.1	59
mc4r	CCCGCGGAATCCTTATTGTG	GATGGTTGACTTTGGGGTGA	NM_173278.1	60

Figure 1.



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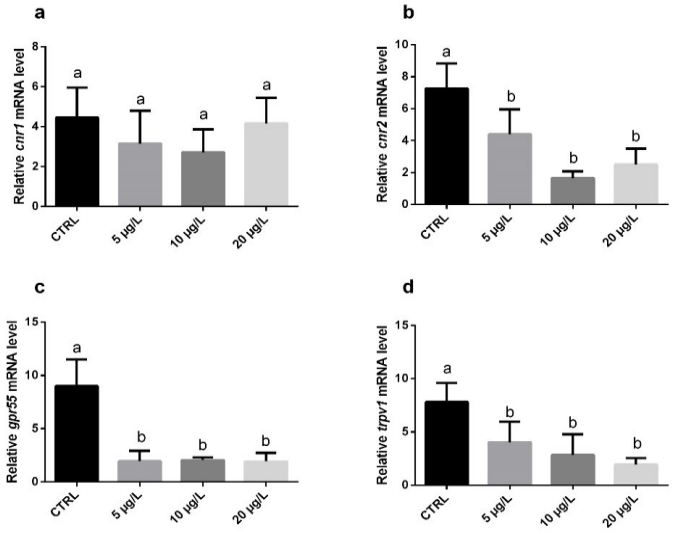

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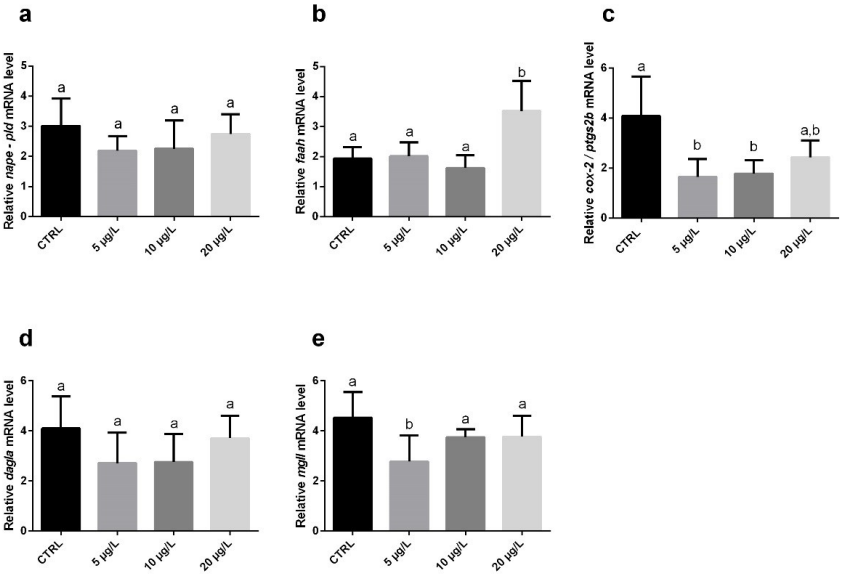
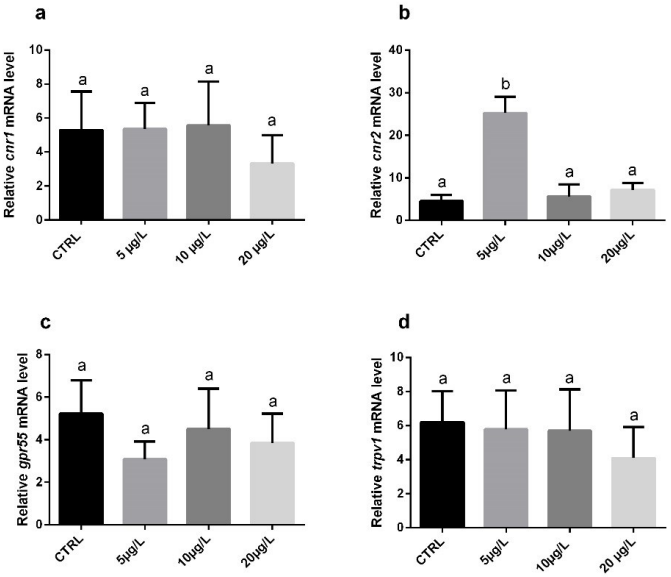


Figure 4.



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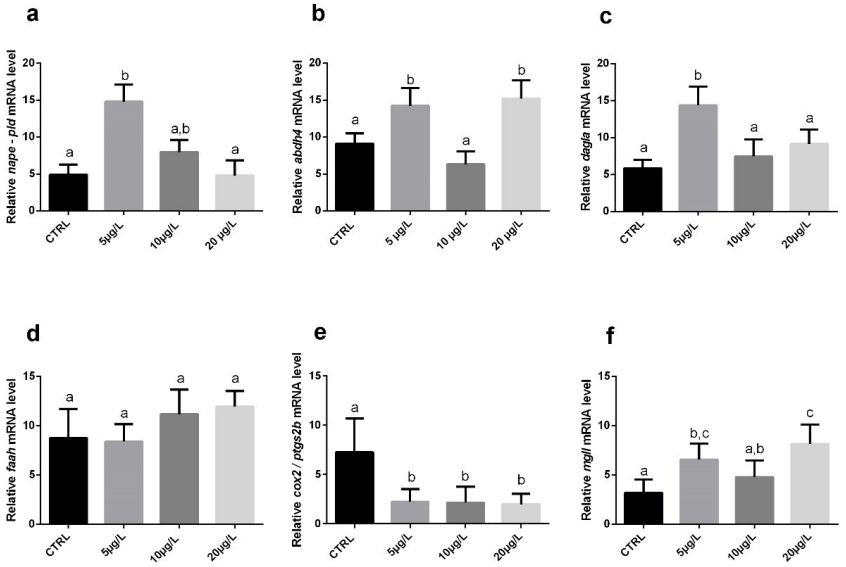

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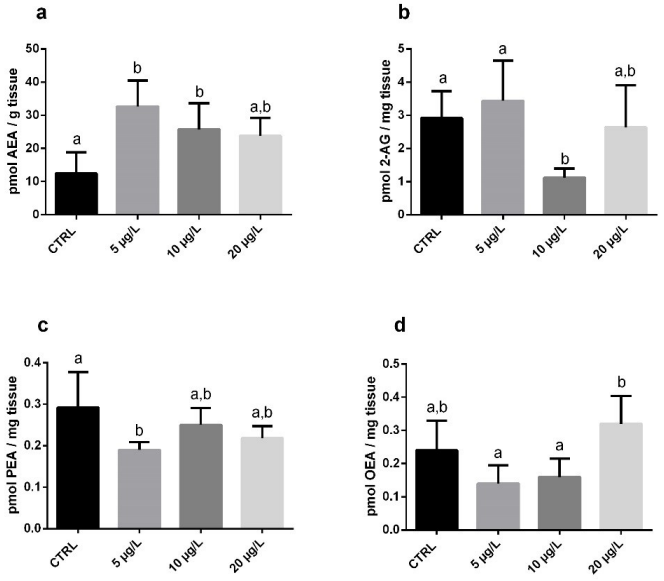


Figure 7.

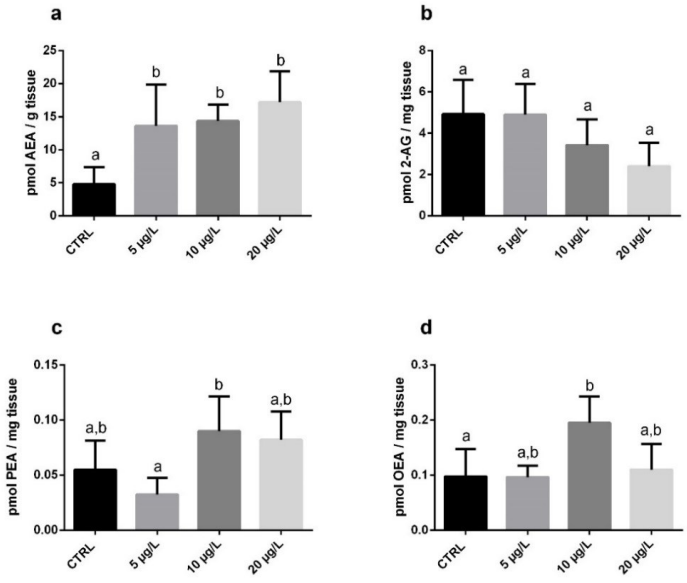
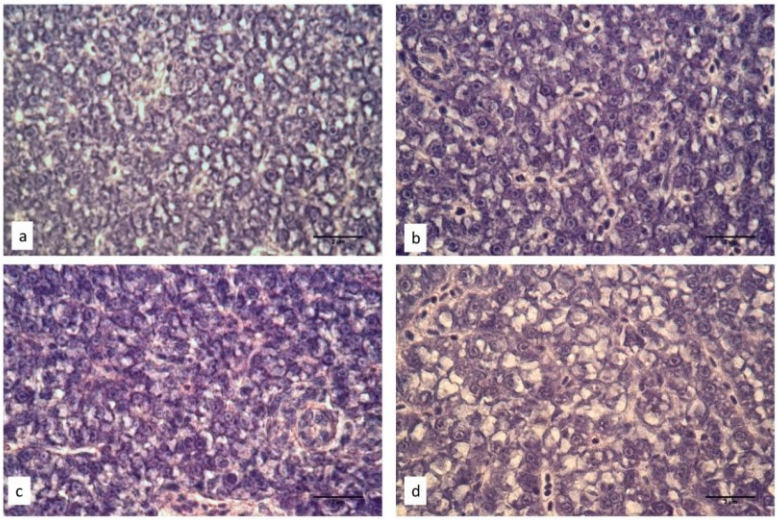


Figure 8.

A)



B)

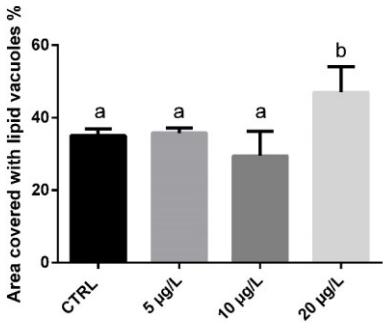
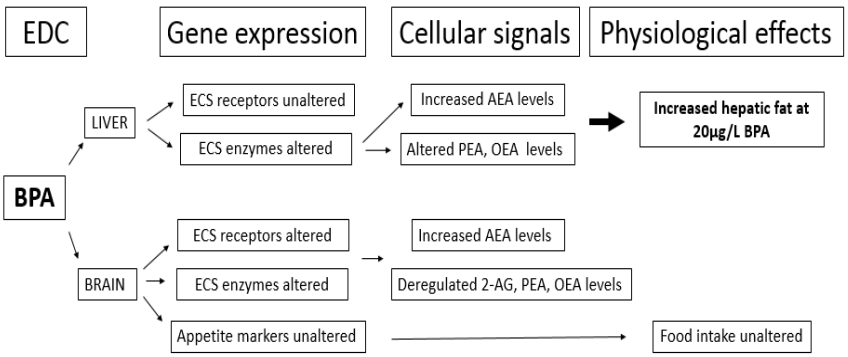

Area covered with lipid vacuoles %
60
40
20
0
a
a
a
b
CTRL
5 µg/L
10 µg/L
20 µg/L

Figure 9.


EDC
Gene expression
Cellular signals
Physiological effects
BPA
LIVER
ECS receptors unaltered
ECS enzymes altered
Increased AEA levels
Altered PEA, OEA levels
Increased hepatic fat at 20µg/L BPA
BRAIN
ECS receptors altered
ECS enzymes altered
Appetite markers unaltered
Increased AEA levels
Deregulated 2-AG, PEA, OEA levels
Food intake unaltered

CHAPTER 3

Effects of Di-isononyl phthalate on zebrafish gonads

Disruption of the gonadal endocannabinoid system in zebrafish exposed to diisononyl phthalate

Forner-Piquer *et al*.

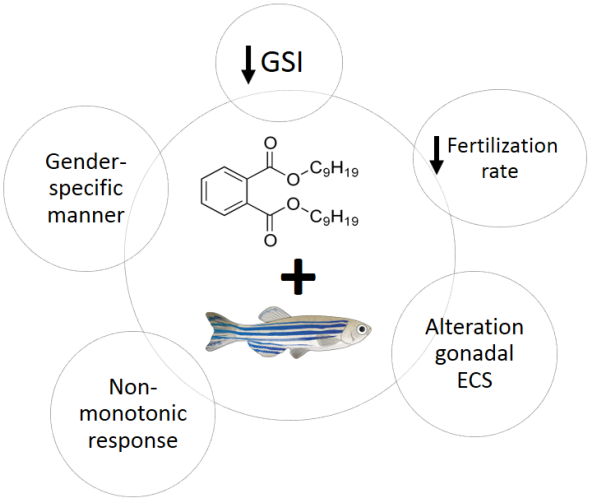
- Manuscript in preparation -

Abstract

DiNP (Di-isononyl phthalate) has been recently introduced as DEHP (Bis (2-ethylhexyl) phthalate) substitute and due to its chemical properties, it is increasingly used in a variety of products. The endocannabinoid system (ECS) is a lipid signaling system involved in a plethora of physiological pathways including the control of the reproductive and metabolic processes. In this study, the effects of DiNP on the ECS of zebrafish (male and female) gonads were analyzed. Adult zebrafish were chronically exposed for 21 days via water to 3 environmentally relevant concentrations (42 µg/L; 4.2 µg/L; 0.42 µg/L) of DiNP. In females, the Gonadosomatic Index (GSI) and the number of fertilized eggs were reduced by the lowest concentration of DiNP tested. The levels of two endocannabinoids, AEA and 2-AG, remained unchanged, while a reduction of OEA levels was measured. Transcriptional changes were reported in relation to genes coding for the ECS receptors and the enzymes involved in the ECS pathway. DiNP exposure in males reduced the GSI as well as changed the levels of endocannabinoids. Moreover, DiNP treatment induced changes in genes coding for the ECS receptors and enzymes, as well as, the enzymatic activity of the fatty acid amide hydrolase (FAAH) was increased after the treatment. In summary, in zebrafish, exposure to environmentally relevant concentrations of DiNP disrupted the ECS and affected reproduction in a gender specific manner.

Capsule: Effects of diisononyl phthalate on the gonadal endocannabinoid system of female and male zebrafish.

Graphical abstract



Highlights:

1. DiNP at environmentally relevant concentrations decreased the fertility rate and the GSI in male and female adult zebrafish.
2. DiNP induced alterations in the ECS at gonadal level in male and female adult zebrafish.
3. DiNP acts in a non-monotonic and in a gender-specific manner in adult zebrafish.
4. ECS as a new target for EDC's exposures.

Key words: DiNP, endocannabinoids, gonads, zebrafish.

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Engineering Research Council of Canada (NSERC) grant to HRH. The authors are grateful to Roberta Verde and Marco Allarà for their technical assistance.

Abbreviations: DiNP: diisononyl phthalate, ECS: Endocannabinoid System, EC: endocannabinoid, EDC: endocrine disruptor chemical, AEA: anandamide, 2-AG: 2-arachidonoylglycerol, PEA: N-palmitoyl-lethanolamide, OEA: N-oleoyl-ethanolamide, CB1: endocannabinoid receptor type I, CB2: endocannabinoid receptor type II, TRPV1: transient receptor potential cation channel subfamily V member 1, GPR55: G coupled protein 55, NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D, FAAH: *Fatty acid amide hydrolase, DAGLα:* Diacylglycerol Lipase alpha*, MAGL:* Monoacylglycerol lipase*, COX-2/ptg2b: cyclooxigenase-2, ABDH4:* α/β-hydrolase 4, ER: estrogen receptor, AR: androgen receptor.

Introduction

Di-isononyl phthalate (DiNP) is a plastic additive from the family of the high molecular weight ortophthalates. Around 95% of its manufacture is used in PVC applications and the rest in non-PVC items. It is found in common household products, as food wrapping, cosmetics, toys, paints, lubricants or adhesives (ECHA, 2010). In 2014, phthalates represented the 70% of the global utilized plasticizers (IHS-Markit, 2015) and as a result, exposure to phthalates increased around the world (Kimber and Dearman, 2010). Phthalates can easily contaminate the environment via losses during the manufacture, sewage or leaching from plastic products. Phthalates, including DiNP, are in fact, not bound to the polymer matrix and can easily be released into the environment (Afshari et al., 2004; Fujii et al., 2003). Reports of phthalate toxicity date back to 1950s (Zarean et al., 2016) and more recent studies provided a better characterization of their adverse health

effects in human and animals, either *in vivo* (Carnevali et al., 2010; Forner-Piquer et al., 2017; Golshan et al., 2015; Howdeshell et al., 2008; Lovekamp-Swan and Davis, 2003; Santangeli et al., 2017) or *in vitro* (Ghisari and Bonefeld-Jorgensen, 2009; Maradonna et al., 2013; Rendel et al., 2017), highlighting their endocrine disrupting properties. Although DiNP is metabolized by animals and humans, usually with a low bioaccumulation or bioconcentration factor (for review Saravanabhavan and Murray, 2012), its abundance it can pose a health problem (Lington et al., 1997; Patyna et al., 2006). In this context, human exposure to DiNP has continuously increased over the years, doubling the median daily intake from 1988 to 2003 (Wittassek et al., 2007).

Recently, the adverse effects of DiNP on zebrafish ovarian function (Santangeli et al., 2017) and a possible involvement of the ECS as target of Endocrine Disrupting Chemicals (EDC) were demonstrated (Forner-Piquer et al., 2017; Martella et al., 2016). In this study, a further investigation on the effects of DiNP on the gonadal Endocannabinoid System (ECS) in male and female zebrafish was carried out.

Briefly, the ECS is a lipid based signaling system localized throughout the body, where the action of endocannabinoids (EC), anandamide (*N*-arachidonoyl-ethanolamine, AEA) and 2-arachidonoyglycerol (2-AG) are mediated by two G-protein-coupled receptors: the endocannabinoid receptor type 1 (CB1) and type 2 (CB2). Commonly, the EC are locally biosynthesized on demand from phospholipid precursors by two enzymes: *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) for AEA and *sn*-1-diacylglycerol lipase α and β (DAGLα and β) for 2-AG. The AEA and 2-AG are inactivated via enzymatic hydrolysis, AEA by the fatty acid amide hydrolase (FAAH) and the 2-AG by the monoacylglycerol lipase (MGLL), (Di Marzo, 2009). It is well established that some endocannabinoids, as AEA, are endogenous ligands of receptors other than CB1 and CB2, such as the transient receptor potential vanilloid type 1 channel (TRPV1) (Ross, 2003) and the G-protein coupled receptor 55 (GPR55) (Ryberg et al., 2007). The AEA biosynthetic

and hydrolytic enzymes are also responsible for the production and inactivation of AEA-related *N*-acylethanolamines, as *N*-palmitoyl-ethanolamine (PEA) and *N*-oleoyl-ethanolamine (OEA), which activate TRPV1, GPR55 and peroxisome proliferator-activated receptors (Fu et al., 2003; Lo Verme et al., 2004; Nagy et al., 2014; Ryberg et al., 2007).

There is evidence that the ECS regulates reproductive functions by acting at multiple levels from gametogenesis to embryo development, in a wide range of species (Battista et al., 2007; Chianese et al., 2011; Grimaldi et al., 2013; Rapino et al., 2014; Wang et al., 2006). In teleost, the cloning of two genes coding for CB1 in the puffer fish, *Fugu rubripes,* by the Yamaguchi team (Yamaguchi et al., 1996) was a milestone in the cannabinoid comparative research; subsequently, basic components of the ECS were characterized in the gonads of bony fish *Carassius auratus* (Cottone et al., 2008) and *Sparus aurata* (Ruggeri et al., 2007). Although it was suggested that zebrafish ECS may have functional roles similar to those of mammals (Krug and Clark, 2015), more research is needed since little is known about the role of ECS in the control of reproduction in non-mammalian vertebrates, including zebrafish (Cottone et al., 2013).

Thus, the main objective of the present study was to investigate the effect of the exposure to environmentally relevant concentrations of DiNP on the ECS in ovary and testes of zebrafish.

Materials and methods

Animals and DINP treatment. The experiments were carried out using one-year old female and male zebrafish (*Danio rerio*, wild-type strain). Fish were maintained in 100-L aquaria with oxygenated water under controlled conditions (28.0 ± 0.5˚C under a 14/10 hours of light/dark period) and fed twice per day with a 1:1 mixture of adult zebrafish complete diet (Zeigler Bross, Inc., Aquamerik Inc., St. Nicolas QC,

Canada) and live brine shrimp nauplii, Artemia cysts. The week before starting the treatment, a large number of fish were crossed randomly in order to ensure to select fish with similar rate of eggs production and fertilization.

Fish were exposed for 21 days in duplicate. Each tank contained 25 females and 25 male zebrafish according to protocols and procedures of the University of Calgary. Three nominal concentrations of DiNP were used: 42 µg/L (10^{-7}M); 4.2 µg/L (10^{-8}M); and 0.42 µg/L (10^{-9}M) (Sigma-Aldrich, Oakville, Canada) diluted with 1mL of 100% EtOH (vehicle). Since the ethanol used as DINP solvent did not exceed the limits set by the Organization for Economic Cooperation Development, the control group was reared only with fresh water (OECD, 2012). Nominal concentrations of DiNP was chosen based on previously studies (Forner-Piquer et al., 2017; Santangeli et al., 2017). DiNP concentrations in water were confirmed by Gas Chromatography – Mass Spectrometry (GC-MS). After twenty-one days of treatment, fish were euthanized with buffered MS-222 (3-aminobenzoic acid ethyl ester) (Sigma-Aldrich, Oakville, Canada) and sampled at 8-10 a.m. to avoid any possible circadian variability. Ovaries and testes from each experimental group were removed and immediately frozen on dry ice and stored at -80ºC for RNA extraction and endocannabinoid assays. In addition, testes and ovaries were fixed in Bouin solution for histology.

Fecundity rate. The bottom of each breeding tank was covered by a grid to allow the embryos to pass into separate spawning trays. In the early morning, spawning trays were removed, embryos were collected and inspected under a stereo microscope to estimate the fertility rate. Fertility was determined from the 14th to the 21st day of the treatment every day. The number of fertilized eggs per female were used to calculate the fertilization rate.

Gonadosomatic Index (GSI) was calculated following the formula: [(gonad weight / total zebrafish weight) x 100].

RNA extraction and cDNA synthesis. Total RNA was isolated from the testes and ovaries with RNAzol solution (Sigma Aldrich, Milan, Italy) according to the manufacturer's instructions and methods described by Santangeli et al., (2016). Final RNA concentrations and integrity were determined as indicated Forner-Piquer et al., (2017).

Real-Time qPCR. The relative quantification of gene expressions was performed as described in Santangeli et al., (2016). Primer list is shown in table 1 (Supplementary Materials) and used at a final concentration of 10 pmol/µL.

Two different genes, namely *rplp0 (*Ribosomal Protein Large P0) and *eef1a1/1* (Elongation Factor 1 alfa 1 like 1), were used as internal controls with the aim of enabling results standardization. Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad) including Genex Macro iQ5 Conversion and genex Macro iQ5 files.

Measurement of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) in testes and ovaries. The extraction, purification and quantification of the levels of endocannabinoids and AEA-like mediators (EC) from the ovaries and testes have been done as previously described (Di Marzo et al., 2001).

FAAH enzymatic activity in ovary and testes. A pool of three ovaries and testes were homogenized per triplicates for each group following (Forner-Piquer et al., 2017).

Ovary and testis morphology. Testis and ovaries were fixed in Bouin and prepared for histological procedures. The tissues were embedded in paraffin and sectioned (4 µm) with a microtome (Leica RM2125 RTS). Each gonad was fully sectioned, stained with Mayer's hematoxylin-eosin and observed under a Zeiss Axio Imager.M2 microscope. Microphotographs were captured using a high-resolution camera Zeiss Axiocam 105 color.

Statistical analysis. All the assays were analyzed by one-way ANOVA followed by Tukey multiple comparison test. Data fulfilled the condition for using a parametric test and when data did not fulfil the criteria for parametric test, Kruskal-Wallis (non-parametric test) was used. Confidence interval was set at 95% ($p < 0.05$). Results are presented as mean ± standard deviation (SD). All statistical procedures were run using *GraphPad Prism 6* and *SigmaStat 3.5.* Letters indicate statistical differences among the treatments and (*) respect the control.

Results

Females **–** Exposure to DiNP decreased the number of fertilized eggs, and the greatest effect was observed at the lowest concentration tested (Figure 1). The GSI was significantly decreased after the exposure to the lowest and middle concentrations of DiNP (Figure 2a). Since the aim was to investigate the effects of DiNP exposure on the ECS in the ovary, we studied the expression of genes encoding for the receptors and the enzymes involved in this signaling pathway.

The transcript for the gene encoding for *cnr1* was increased following exposure to 0.42 µg/L and 4.2 µg/L DiNP, however no changes were reported for the *cnr2*. Controversially, *gpr55* transcript level was significantly increased following exposure to the highest concentration of DiNP, while the level of *trpv1* was also increased, but statistically significant only at 4.2 µg/L as it can be observed in Table 1.

For the enzymes involved in biosynthesis of AEA, as it is shown in Table 1, transcript level for *nape-pld* and *abdh4* were significantly increased following exposure by the lowest and medium concentrations of DiNP. Also, *faah* followed the same expression pattern as *abdh4*, being increased by exposure to 0.42 and 4.2 µg/L of DiNP. Regarding 2-AG enzymes, the level of mRNA for the biosynthetic enzyme *daglα* was increased by the lowest concentration, while the level of the catalytic

enzyme *mgll* mRNA was significantly increased following exposure to 4.2µg/L of DiNP. The highest DiNP concentration, 42 µg/L, did not affect the transcript levels for these enzymes. Finally, DiNP exposure did not alter the transcript levels for *cox-2/ptgs2b,* enzyme involved in the conversion of AEA and 2-AG into oxidation products as part of inflammatory response.

Considering the modulation of the ECS signals, the levels of the EC and EC-like mediators were evaluated in the ovaries (Table 3a). Neither AEA levels, nor FAAH activity varied in the ovary of females exposed to DiNP, (Figure 3). Regarding the other main cannabinoid, the level of 2-AG was decreased following exposure with 4.2µg/L DiNP. No change in PEA levels was observed, while the OEA ones decreased.

Histological analysis in the ovaries did not show morphological alterations following exposure with DiNP (Figure 4).

Males – In male fish, GSI was decreased following exposure to DiNP (Figure 2b). As performed for females, the genes encoding for the ECS pathway were analyzed. Transcript levels for the *cnr1* and *cnr2* were reduced following exposure to the lowest and medium concentrations of DiNP, and increased at the highest concentration tested as it can be observed in Table 2. Similarly, the transcript levels for the genes coding for *gpr55* and *trpv1* were reduced following exposure to the lowest and medium concentrations of DiNP. Regarding the transcripts of ECS enzymes illustrated in Table 2, the mRNA levels of *nape-pld* was reduced following exposure to 0.42 µg/L of DiNP, whilst the levels of *abdh4* remained unchanged. The levels of the transcript coding for the biosynthetic 2-AG enzyme, *daglα*, were decreased following exposure with all concentrations of DiNP. mRNA levels for the hydrolytic enzyme *faah* were markedly decreased following exposure to all concentrations of DiNP, while for the main catabolic enzyme of 2-AG, *mgll*, DiNP significantly reduced its transcript levels only at the 0.42 µg/L concentration. Finally, the expression of the gene coding for *cox-2/ptgs2b* was significantly

reduced following exposure with 0.42 and 4.2 µg/L DiNP. In addition, the mRNA levels of the estrogen receptor alfa (*esr1*), estrogen receptor beta subunit 2 (*esr2b*), and androgen receptor (*ar*) were reduced in all the DiNP treated groups.

Regarding the endocannabinoids and EC-like mediator levels were also measured in the testes (Table 3b). Exposure to all concentrations of DiNP increased AEA level, although the differences were only significant at 0.42µg/L. FAAH enzymatic activity was increased following exposure with 0.42 and 4.2 µg/L DiNP, but not at the highest concentration tested (Figure 3b). On the contrary, exposure at all three DiNP concentrations reduced the level of the 2-AG with respect to control. Finally, the exposure to 0.42 and 42 µg/L DiNP increased the EC-like mediator PEA and decreased OEA level.

Histological studies revealed that in all experimental groups, the testes contained spermatozoa with no apparent pathological alterations at the macrostructural level, and no clear evidence of disruption by DiNP (Figure 5).

Discussion

In rats perinatally exposed to high concentrations of DiNP, histological changes in fetal testes with reduced testosterone level and lower percentage of motile sperm were reported (Boberg et al., 2011). DiNP was also linked with altered sexual differentiation and comorbidities (Gray et al., 2000). Furthermore, there is evidence that DiNP possess anti-androgenic properties in both male rats and zebrafish embryos (Chen et al., 2014; European Chemicals Agency, 2012), but with lower potency than other phthalates (Boberg et al., 2011; Lee and Koo, 2007). Then, since the possible interaction of DiNP with the ECS in the reproductive organs is still an unexplored issue, the main objective of the present study was to evaluate the effect of environmentally relevant concentrations of DiNP in zebrafish gonads, focusing on the modulation of the ECS signaling.

The ECS has emerged as one of the main signaling system in both female and male reproduction (Maccarrone, 2009). There is evidence that the ECS is involved in human and other mammalian reproductive processes, ranging from oogenesis/spermatogenesis to embryo and placental development, indicating that a correct tone of endocannabinoids is critical for a normal reproductive outcome (Battista et al., 2012; Taylor et al., 2007; Wang et al., 2006). To date, most of the studies regarding the role of the ECS in reproductive performance are focused on mammals, and only few reports exist regarding the presence and the functionality of ECS in teleost gonads (Cottone et al., 2008; Ruggeri et al., 2007).

The results presented in the study, clearly pinpointed that DiNP reduced the fertility rate and the GSI, more dramatically in males than female. Similar differences were observed in other teleost fish exposed to DEHP (Chikae et al., 2004) or diethyl phthalate (DEP) (Barse et al., 2007). Regarding the ovarian ECS, DiNP weakly affected AEA levels, probably due to a compensation between the upregulation of the genes coding for its biosynthetic (*nape-pld* and *abdh4*) and inactivating (*faah)* enzymes, which is consistent with the unaltered FAAH activity. In humans, a role for AEA has been suggested in oocyte maturation and ovulation process (El-Talatini et al., 2009, Fonseca et al., 2010), while in teleosts the role of AEA remains unknown.

Despite AEA, OEA and PEA are hypothesized to be biosynthesized in the granulosa cells, but the mechanism controlling their production and release is still not known (Taylor et al., 2007). Nevertheless, they have been found in the follicular fluids of human and mouse, leading to the hypothesis that *N*-acylethanolamine signaling (PEA, OEA and AEA) may help to regulate follicular maturation and development (Schuel et al., 2002). In addition, OEA has been suggested to act as an anti-inflammatory mediator (Suardíaz et al., 2007). Therefore, a decrease in OEA level together with an increase in *trpv1* transcript level (and unchanged *cox-2*) in the ovary could be an indication of an inflammatory response in the gonads of DiNP

exposed fish. Concerning 2-AG, its levels were significantly reduced only by 4.2 µg/L DiNP treatment, possibly in association with the upregulation of the gene coding for the main 2-AG catabolic enzyme, *mgll*, and unchanged levels of the biosynthetic enzyme *dagla* transcript. To date, 2-AG has been widely described in the mammalian uterus (Fonseca et al., 2010), but its role in teleost reproductive processes is still unknown.

As mentioned above, DiNP upregulated the *cnr1* mRNA levels at the lowest and middle concentrations. In mice, CB1 is needed for oocyte maturation. Indeed, the absence of CB1 during oocyte maturation led to impaired reproductive process (López-Cardona et al., 2017). However, the role of CB1 in fish oocytes may be different from that in mammals. While the presence of CB1 has been described in the teleost ovary (Ruggeri et al., 2007), its function remains unclear. Furthermore, as it has been previously reported (Santangeli et al., 2017) that the same DiNP concentrations, unaffected the expression of signals involved in oocyte maturation, including *lhcgr, pgrmc1* and *pgrmc2*. In addition, as Santangeli and colleagues reported in their study, the frequency of mature oocytes in treated females did not change (Santangeli et al., 2017).

Regarding males, higher concentrations of AEA were found in the testes of DiNP treated zebrafish, which is consistent with the observed downregulation of *faah* and *cox-2*. However, the biosynthetic enzymes of AEA, *nape-pld* and *abdh4*, remained unaltered, or slightly reduced. However, lack of correlation was found between the expression of *faah* and FAAH activity, which appears to be contradictory with respect to the observed increase in AEA level in the testes of treated fish. It should be noted that AEA-like mediators, such as OEA and PEA, also act as “entourage compounds” (De Petrocellis et al., 2001; Jonsson et al., 2001). This means that OEA and PEA may enhance the levels of AEA inhibiting its degradation by FAAH acting as alternative substrates. On this regard, OEA levels were reduced following exposure with 0.42 and 4.2 µg/L DiNP, suggesting that this

compound may have been hydrolyzed by an increased FAAH activity instead of AEA. In addition, PEA can decrease *faah* expression, thus potentiating AEA effects (Di Marzo et al., 2001), which is consistent with the observed increase in PEA levels and the concomitant downregulation of *faah* in all DiNP treated groups. Thus, "entourage effects" by OEA and PEA might explain why AEA levels were increased and *faah* transcript levels decreased by the lowest and middle concentrations of DiNP despite the stimulatory effect of the middle dose on FAAH activity.

It has also been reported that estradiol (E_2) can increase *faah* mRNA levels in Sertoli Cells (Grimaldi et al., 2012; Rossi et al., 2007) and that the *faah* promoter contains imperfect Estrogen Response Elements (EREs) (Waleh et al., 2002), mediating E_2 effects through ERβ on the promoter activity in mouse Sertoli cells (Grimaldi et al., 2012). In addition, CB1 mRNA can be modulated by E_2, adding further evidence of interaction among steroids and ECS (Laprairie et al., 2012 for review). Hence, downregulation of genes such as *faah* and *cnr1*, on one hand, and *esr1* and *esr2*, on the other hand, might be inter-related due to stimulatory effects of estrogens on ECS genes. These observations together with a decreased GSI and *ar* levels, indicate that environmental concentrations of DiNP may disrupt zebrafish male reproduction by altering the endocrine/paracrine control mechanisms. These observations are in agreement with previous results in mammals where higher DiNP concentrations exerted anti-androgenic activity, as suggested by the European Chemical Agency (ECHA, 2013).

There is evidence that ECS components are involved in multiple steps of the reproductive processes. For example, AEA, next to be involved in apoptosis of Sertoli cells (Maccarrone et al., 2000), is also involved in sperm motility, and can influence fertility in different species (Berdyshev, 1999; Cobellis et al., 2006; Maccarrone et al., 2005; Schuel and Burkman, 2006). In the frog, *R. esculenta*, AEA is associated with reduced sperm motility (Cobellis et al., 2006) and lower levels of testosterone (Wenger et al., 2001). Furthermore, AEA, via CB1 or CB2, increases

the proportion of slow or immobile sperm (Agirregoitia et al., 2010; Cobellis et al., 2006). These findings provide a support for the hypothesis that high levels of AEA in combination with dysregulation of *cnr1* and *cnr2* in the DiNP-treated zebrafish may alter sperm motility, and consequently reduce the fertility rate.

The observed decrease of the 2-AG levels in testes could be related to downregulation of the main biosynthetic enzyme *dagla*. Mitotic male germ cells express high levels of 2-AG that, by activating CB2, promotes the progression of spermatogonia towards spermatogenesis (Grimaldi et al., 2009). In DiNP treated male zebrafish, the observed overexpression of *cnr2* at the highest concentration might represent an adaptive response to the lowest levels of 2-AG, which in turn would mediate a DiNP-induced blockade of spermatogenesis. However, additional studies are needed to support this hypothesis.

In conclusion, the results obtained here following a chronic exposure of adult zebrafish to environmentally relevant concentrations of DiNP provide evidences for the hypothesis that DiNP affects reproductive processes, in part, by disrupting the ECS in zebrafish. The observed disruption by DiNP on endocannabinoid signaling was found to be non-monotonic and gender-specific, exerting greater disruption on male than on female. The overall findings provide a framework for a better understanding of the disruptive effects of DiNP on reproduction and a contribution in the Adverse Outcome Pathway (AOP) (Figure 6) of DiNP for the assessment of its mechanism of action.

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Figures & tables

Table 1. Transcriptional profiles of the ECS pathway in the ovaries expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5 manager software. Letters above each column indicate statistical differences among groups (one-way ANOVA followed by Tukey's multiple comparison test; $p < 0.05$).

Table 2. Transcriptional profiles of the ECS pathway, estrogen receptors (*esr1*, *esr2b*) and androgen receptor (*ar*) in testes expressed as relative mRNA abundance ± SD and determined by Bio-Rad Laboratories iQ5 manager software. Letters above each column indicate statistical differences among groups (one-way ANOVA followed by Tukey's multiple comparison test; $p < 0.05$).

Table 3. Levels of Anandamide (AEA); 2-arachidonoylglycerol (2-AG); *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA) in the ovaries (A) and testes (B) of zebrafish treated with different concentrations of DiNP. Asterisks (*) indicate significant differences between the treatment and control group ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$). Evaluated by one-way ANOVA with Tukey post – test. Levels as means ± SD of pmol/mg wet tissue or pmol/g wet tissue.

Figure 1. (A) Fertilization rate as number of fertilized eggs per female zebrafish per day. Letters above each column indicate statistical differences among groups ($p < 0.05$; one - way ANOVA followed by Tukey's multiple comparison test).

Figure 2. Gonadosomatic index of females (a) and males (b). Letters above each column indicate statistical differences among groups ($p < 0.05$; one - way ANOVA followed by Tukey's multiple comparison test).

Figure 3. FAAH enzymatic assay in ovaries (a) and testes (b). Data reported as pmol/min/mg and expressed as means ± SD. Letters above each column indicate

statistical differences among groups ($p < 0.05$; one – way ANOVA followed by Tukey's multiple comparison test).

Figure 4. (A) Histological sections of ovary stained with Mayer's hematoxylin-eosin. Scale bar: 200 µm. Control (a); 0, 42 µg/L (b); 4, 2 µg/L (b); 42 µg/L (d).

Figure 5. Histological sections of testis stained with Mayer's hematoxylin-eosin. Scale bar: 200 µm. Control (a); 0, 42 µg/L (b); 4, 2 µg/L (c); 42 µg/L (d).

Figure 6. Adverse Outcome Pathway (AOP) for DiNP in male and female zebrafish gonads treated with environmental concentrations of DiNP.

Table 1.

(A)

	AEA (pmol/g)	2-AG (pmol/mg)	PEA(pmol/mg)	OEA(pmol/mg)
OVARY				
CONTROL	15.92 ± 3.71	3.08 ± 1.03	0.10 ± 0.03	0.21 ± 0.06
0.42µg / L	14.80 ± 3.42	3.96 ± 1.15	0.15 ± 0.04	0.13 ± 0.05
4.2µg / L	17.08 ± 4.01	0.77 ± 0.22 **	0.12 ± 0.03	0.01 ± 0.00 ***
42µg / L	21.90 ± 3.49	3.18 ± 0.77	0.12 ± 0.02	0.02 ± 0.01 ***

(B)

	AEA (pmol/g)	2-AG (pmol/mg)	PEA(pmol/mg)	OEA(pmol/mg)
TESTIS				
CONTROL	23.18 ± 5.34	6.86 ± 2.24	0.10 ± 0.04	0.09 ± 0.03
0.42µg / L	43.64 ± 12.93	4.06 ± 0.94 *	0.19 ± 0.05 *	0.04 ± 0.02 *
4.2µg / L	108.9 ± 14.96 ****	3.82 ± 1.19 *	0.17 ± 0.04	0.04 ± 0.02 *
42µg / L	89.63 ± 19.8 ****	3.60 ± 1.91 *	0.19 ± 0.04 *	0.10 ± 0.03

Table 2

GENE	CONTROL	0.42 µg/L DiNP	4.2 µg/L DiNP	42 µg/L DiNP
cnr1	1.99 ± 0.91 [a]	4.28 ± 1.23 [a]	5.32 ± 1.62 [b]	2.53 ± 1.50 [a]
cnr2	1.41 ± 0.44 [a]	1.68 ± 0.25 [a]	2.25 ± 0.92 [a]	1.67 ± 0.56 [a]
gpr55	11.25 ± 4.23 [a]	7.32 ± 0.95 [a,b]	10.58 ± 3.46[a,b]	3.48 ± 2.18 [b]
trpv1	2.12 ± 0.69 [a]	4.08 ± 2.28 [a]	11.47 ± 2.71 [b]	3.36 ± 2.48 [a]
nape-pld	3.07 ± 1.06 [a]	6.08 ± 1.59 [b]	3.73 ± 1.33 [a,b]	2.16 ± 0.69 [a]

abdh4	1.97 ± 0.76 [a]	14.52 ± 3.71 [b]	13.08 ± 4.15 [b]	2. 89 ± 1.31 [a]
faah	4.81 ± 0.92 [a]	37.58 ± 18.41[b]	55.12 ± 13.28[b]	8.07 ± 5.68 [a]
dagla	2.49 ± 1.17 [a]	9.32 ± 3.91 [b]	4.01 ± 2.01 [a]	2.13 ± 1.65 [a]
mgll	3.18 ± 0.29 [a,b]	7.53 ± 4.20 [a,b]	10.21 ± 4.22 [b]	2.30 ± 1.13 [a]
cox2/ptg2b	3.41 ± 0.87 [a]	5.38 ± 1.71 [a]	4.84 ± 1.57 [a]	4.18 ± 1.47 [a]

Table 3

GENE	CONTROL	0.42 µg/L DiNP	4.2 µg/L DiNP	42 µg/L DiNP
cnr1	11.65 ± 0.57 [a]	3.12 ± 1.40 [b]	1.92 ± 1.30 [b]	24.82 ± 5.04 [c]
cnr2	13.16 ± 13.87 [a]	10.86 ± 3.19 [a]	8.37 ± 0.07 [a]	54.17 ± 11.09[b]
gpr55	9.42 ± 1.58 [a]	3.56 ± 1.75 [b]	2.30 ± 1.83 [b]	8.27 ± 1.06 [a]
trpv1	4.89 ± 0.60 [a]	1.99 ± 0.80 [b]	1.28 ± 0.04 [b]	4.74 ± 1.66 [a]
nape-pld	4.24 ± 1.01 [a]	4.12 ± 1.09 [a]	1.50 ± 0.71 [b]	2.93 ± 0.82 [a,b]
abdh4	4.55 ± 1.75 [a]	3.76 ± 1.32 [a]	5.66 ± 1.90 [a]	4.84 ± 2.07 [a]
faah	11.77 ± 3.59 [a]	3.82 ± 2.20 [b]	4.73 ± 2.45 [b]	2.15 ± 0.42 [b]
dagla	6.25 ± 1.95 [a]	1.79 ± 0.53 [b]	1.63 ± 0.71 [b]	1.96 ± 0.38 [b]
mgll	3.86 ± 0.91 [a]	2.48 ± 0.57 [a,b]	1.54 ± 0.76 [b]	3.52 ± 1.06 [a,b]
cox2/ptg2b	5.31 ± 1.05 [a]	3.14 ± 0.64 [b]	1.44 ± 0.62 [b]	5.67 ± 1.17 [a]
esr1	6.45 ± 1.94 [a,b]	3.27 ± 1.34 [b,c]	1.31 ± 0.44 [c]	2.49 ± 1.48 [c]
esr2b	9.67 ± 2.95 [a]	1.92 ± 1.01 [b]	1.59 ± 0.13 [b]	1.81 ± 1.29 [b]
ar	14.69 ± 5.94 [a]	2.17 ± 1.14 [b]	2.17 ± 1.32 [b]	3.18 ± 2.16 [b]

Figure 1

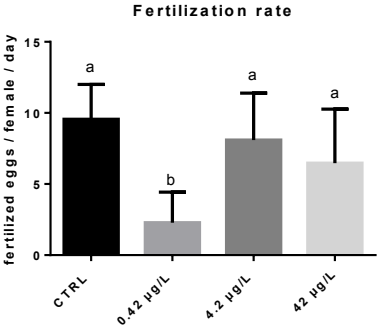


Figure 2.

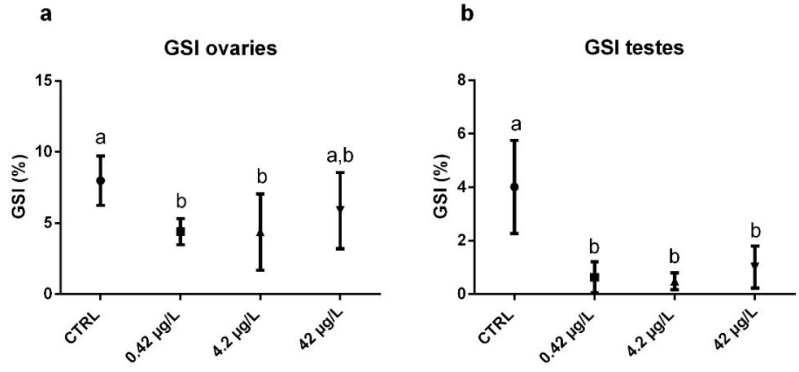


Figure 3

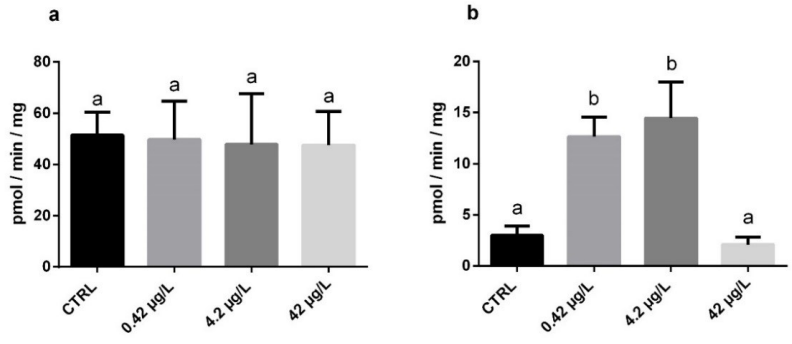


Figure 4

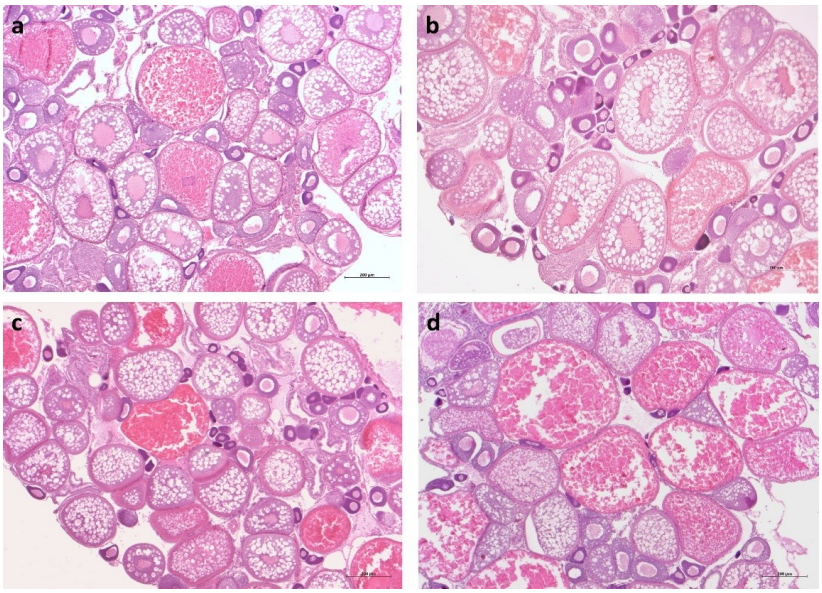


Figure 5.

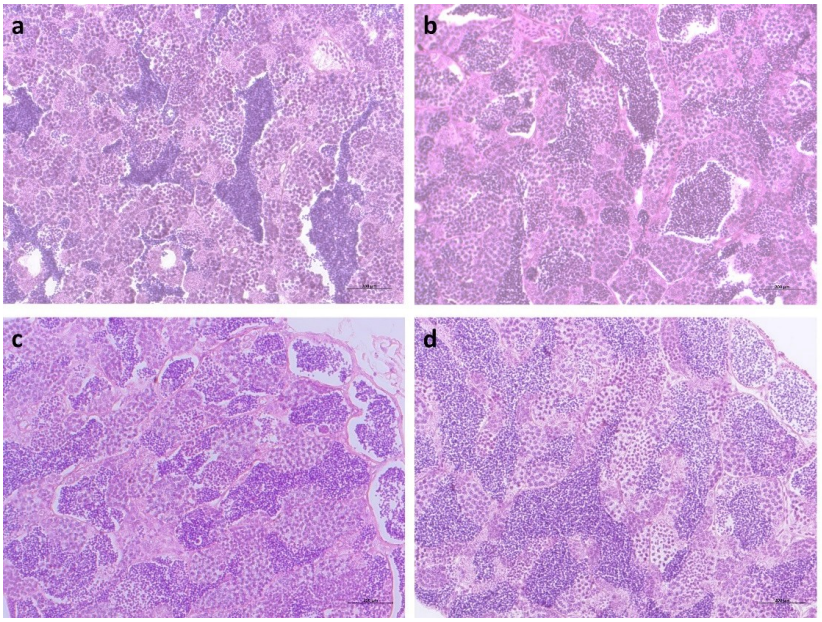
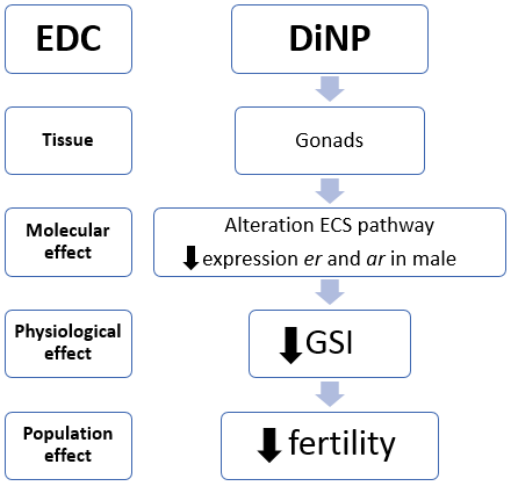


Figure 6


EDC
DiNP
Tissue
Gonads
Molecular effect
Alteration ECS pathway
⬇expression *er* and *ar* in male
Physiological effect
⬇GSI
Population effect
⬇fertility

Supplementary material. Table 1. Primer list. *rplp0*: ribosomal protein large P0; *eef1a/1:* elongation factor 1 alfa 1 like 1; *cnr1:* endocannabinoid receptor type I; *cnr2:* endocannabinoid receptor type II; *dagla:* diacylglycerol lipase alpha; *mgll:* monoglyceride lipase; *nape-pld:* N-acyl phosphatidylethanolamine phospholipase D; *faah*: fatty acid amide hydrolase; *abdh4*: a/b hydrolase 4; *gpr555:* G protein-coupled receptor 55; *trpv1:* transient receptor potential cation channel subfamily V member 1; *cox-2/ptgs2b:* cyclooxygenase 2b.

Table 1.

Primer	Forward (5'-3')	Reverse (5'-3')	Ta (ºC)	Accession number
rplp0	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	60	NM_131580.2
eef1a1	GACAAGAGAACCATCGAG	CCTCAAACTCACCGACAC	60	NM_131263.1
cnr1	TCTGTGGGAAGCCTGTTTC	ACCGAGTTGAGCCGTTTG	55	NM_212820.1
cnr2	CCATCGTACCTGTTCATTAG	GCTGTCAGCAAAAGGCTTC	58	BC163057.1
gpr55	AACTGAAGGTGTGGATAC	ACGCCATAATGTTTAACG	53	XM_005163567.3
trpv1	TGATCGTCGCTGGTGCTT	GACTGGGCTCTCTCTGAACG	59	NM_001126399.1
daglα	GAGGGTTTCCGTCGTCAC	TGTTCCTCCAGCAATGATCC	59	XM_692781
mgll	AAGTGAAGGTGAGAGGAT	AATGTCCAACGATGAAGA	53	NM_200297
nape-pld	CTCAAGGACATGCACTCA	GAGCACAATCTTCAAGACAAT	57	NM_001080613
abhd4	GAAGAGCAGTTTGTTTCCTCCATAG	GACTCACTCTTTCTGGGTATTGGAT	60	NM_001017613.1
faah	CATTCTCGGACCAGCCTTCA	CCTTCACTGCCTGTTGGAAGA	60	NM_001109825.1
cox-2 /ptgs2b	ATCCAGGATGAAGTCTACAAT	GCTGTTGACGCCATAATC	57	NM_001025504
esr1	GGTCCAGTGTGGTGTCCTCT	AGAAAGCTTTGCATCCCTCA	58	XM_009299438.3
esr2b	TTGTGTTCTCCAGCATGAGC	CCACATATGGGGAAGGAATG	58	NM_174862.3
ar	ACTGGGACCGAATAAAGCCC	ATGTAATCGCAGCCGGAGAC	58	EF440290.1

CHAPTER 4

Effects of the Di-isononyl phthalate and Bisphenol A on the liver and brain of gilthead sea bream

Endocrine disruptors in the diet of male *Sparus aurata*: modulation of the endocannabinoid system at hepatic and central level by Di-isononyl phthalate and Bisphenol A

Forner-Piquer *et al*.

- *Manuscript in preparation* -

Abbreviated Title: Effects of DiNP and BPA in *Sparus aurata*.

Key words: DiNP, BPA, endocannabinoids, liver, sea bream

Highlights:

1. BPA and DiNP induce morphological and biochemical alteration in the liver.
2. BPA and DiNP alter the levels of EC and EC-like mediators in the liver and brain.
3. BPA and DiNP modify the expression of genes coding for the triglyceride biosynthesis pathway.

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Abstract

The increasing manufacture of plastics and their mismanagement has turn plastic into a ubiquitous waste in the marine environment. Among all the substances conforming the plastic items, the effects of a dietary Bisphenol A (BPA) and Di-isononyl phthalate (DiNP) have been evaluated in adult male gilthead sea bream, focusing on their effects in the modulation of Endocannabinoid System (ECS). ECS has been recently chosen as a new target for the activity of some Endocrine Disruptors Chemicals (EDC), being the cannabinergic pathway a complex lipid signaling network essential for the well-being of the organisms. The results obtained showed that BPA and DiNP altered the hepatic structure and the hepatic biochemical composition, increasing the presence of lipids and triglycerides and decreasing the glycogen and phospholipids. In addition, the presence of BPA or DiNP in the gilthead sea bream diet altered the levels of endocannabinoids (EC) and EC-like mediators in the liver. These alterations were also associated to changes at transcriptomic level of genes involved with the lipid biosynthesis and with the ECS metabolism. At central level, both BPA and DiNP reduced the *cnr1* and *npy* transcription as well as the levels of Anandamide (AEA), suggesting a downregulation of appetite. The results herein reported highlighted the negative effects of the chronic dietary exposure to DiNP and BPA on the ECS functions and the lipid metabolism of male gilthead sea bream liver.

Introduction

Since 1964, world plastic manufacture has increased 20-fold reaching 311 million tons in 2014 (Plastics Europe, 2010). Unfortunately, associated with that, it was estimated that between 4.8 to 12.7 million tons entered to the ocean in 2010, corresponding to the 1.7 to 4.6% of total plastic waste generated in coastal countries (Jambeck et al., 2015) and it is expected by 2050 that our planet will hold

about 33 billion tons of plastic (Rochman et al., 2013). In this line, plastic debris have been found in the stomachs of over 220 different marine species (FAO, 2017).

For years, plastic has been considered as biochemically inert (Lithner et al., 2011), however plastic additives or "plasticizers" may leach out transferring to the marine environment (Rani et al., 2015) and/or introducing potentially hazardous chemicals to biota since plastics are a mixture of chemicals and a platform to accumulate other nearby toxic components (Bakir et al., 2014; Cole et al., 2011; Engler, 2012; Fossi et al., 2014, 2012; Rochman et al., 2014; Yamashita et al., 2011).

Among the myriad of substances used for plastic production, our emphasis will be placed on two plastic additives, the Bisphenol A (BPA) and a phthalate, the Di-isononyl phthalate (DiNP).

Generally, phthalates are chemical compounds primarily used for the manufacture of polyvinyl chloride (PVC), characterized by their incapacity to be chemically bound to plastic matrix (Fujii et al., 2003; Roh et al., 2013). In this regard, DiNP can be found in freshwater, marine water (Blair et al., 2009; Mackintosh et al., 2006, 2004) and sediments (Oehlmann et al., 2008). Nevertheless, phthalates are considered to be no persistent chemicals, to possess a short half-life and a lower bioconcentration factor in fishes than expected (Staples et al., 1997). However, concerns about their toxicity exist due to their potential chronic effects on kidney and liver, since the liver is one of the most sensitive organs for the DiNP toxicity (Ma et al., 2014).

On the other hand, BPA is mainly used in the manufacture of polycarbonate plastics (66%) and epoxy resins (30%). Similarly to DiNP, BPA is considered a no persistent chemical with a short half-life (Staples et al., 1998), however; its presence in a multitude of products turns BPA into a ubiquitous chemical found in all the environmental compartments (Flint et al., 2012; Staples et al., 1998). Release in the environment can take place during the manufacture and consumption via wastewater discharge, landfill leachate or natural breakdown from plastics (Crain

et al., 2007). Despite BPA presents a low potential of bioaccumulation in aquatic organisms (Staples et al., 1998), studies on wildlife indicate that BPA may have negative effects even at environmentally relevant concentrations which is globally set at 12 μg/L. (Crain et al., 2007; Flint et al., 2012; Lahnsteiner et al., 2005; Mandich et al., 2007; Sohoni et al., 2001, Santangeli et al., 2016).

BPA, as well as DiNP, has been firstly reported for their endocrine disrupting properties at reproductive levels and lately, for inducing alterations in the Endocannabinoid System (ECS) (Forner-Piquer et al., 2017; Martella et al., 2016). In this context, the ECS is a lipid signaling network involved in a plethora of physiological roles, as the regulation of energy homeostasis and food intake (Cristino et al., 2014; Di Marzo et al., 2001; Watkins and Kim, 2015) at central (hypothalamus) and peripheral level (liver). Briefly, the ECS is composed by two G-protein coupled receptors: the cannabinoid receptor type I (CB1) and type II (CB2); their endogenous agonist, the endocannabinoids (EC): N-arachidononyl-ethanolamine (Anandamide or AEA) and 2-Arachidononyl-glycerol (2-AG), and two AEA-related compounds (or endocannabinoid-like mediators): N-oleolethanolamine (OEA) and N-palmitoylethanolamine (PEA) regulated by a complex enzymatic machinery composed by biosynthetic and catabolic enzymes. A correct tone of the ECS is fundamental for the well-being and well-performance of the organism, being the deregulation of ECS tone the starting point of several metabolic disorders (Osei-Hyiaman et al., 2008, 2005; Piccinetti et al., 2010).

On the other hand, given the low aqueous solubility of DiNP (and the moderate solubility for BPA) is expected the diet as a relevant route of exposure for wildlife (Staples et al., 1998; Thomann, 1989), as well as, as Bjerregaard reported (Bjerregaard et al., 2007), the possibility that the diet may represent a source for EDC exposure has been so far practically neglected. Thus, based in previous studies performed in gilthead sea bream (Carnevali et al., 2017; Maradonna et al., 2015, 2014), the main goal of the present study was to elucidate whether the dietary

administration of DiNP and BPA might alter the lipid metabolism and the ECS in *Sparus aurata,* using the ECS as a target for EDCs exposures.

Material and methods

Fish maintenance. Two-year-old gilthead sea bream males (mean weight 458.8 ± 56.8 g; Total length 301.7 ± 10.7 cm) were maintained at the AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Centre for Marine Research (HCMR), Iraklion, Crete, Greece. The fish were maintained in 2-m^3 tanks supplied with shallow well-water under ambient photoperiod and stable temperature ranging between 19-20°C. Measurements of dissolved oxygen, pH and NH_3-N and NO_2-N were done weekly during the maintenance period. The fish were fed to apparent satiation twice per day with commercial feed (IRIDA SA, Greece) during the three-month acclimation.

Experimental design. The experimental feed containing the EDCs was prepared according to Bjerregaard et al. (2007) and Maradonna et al. (2014) using the commercial feed that the fish were used to be eating prior to the experiment. The amount of DiNP and BPA added to the feed were adjusted to give the experimental dose according to the feed ration of fish of that size and at the ambient water temperatures. Fish were divided in 5 treatment groups of 10 individuals in duplicates and were stocked in separate 2-m^3 tanks as described earlier and were maintained for 21 days. Fish were fed 0.7% of their body weight (bw), divided to five feedings per day, in order to ensure all the feed administered was consumed. The treatments were as follows: Control individuals (CTRL) were fed with the commercial feed mixed with the phthalate vehicle (1,4 ml of EtOH kg^{-1} feed); BPA LOW were fed with commercial feed enriched with BPA so that fish consuming their daily ration of feed would receive 4 µg BPA kg^{-1} bw day^{-1}; BPA HIGH were fed with commercial feed enriched with BPA so that fish consuming their daily ration of feed would receive 4000 µg BPA kg^{-1} bw day^{-1}; DiNP LOW were fed with

commercial feed enriched DiNP, so that fish consuming their daily ration of feed would receive 15 μg DiNP kg^{-1} bw day^{-1}; DiNP HIGH were fed with commercial feed enriched with DiNP so that fish consuming their daily ration of feed would receive 1500 μg DiNP kg^{-1} bw^{-1}.

The doses chosen for the BPA and DiNP were based on the Tolerable Daily Intake (TDI) for humans ruled by the European Food Safety Authority, which is 4 μg kg^{-1} day^{-1} and 150 μg kg^{-1} day^{-1} respectively. The experimentation was done in the same conditions (tanks, temperature and photoperiod) than the previous acclimation of the animals. Water pH (7,52 ± 0.02) and dissolved oxygen levels (89.2 ± 1.8%) were monitored daily during the experimentation. The water NH_3–N and NO_2–N levels were measured weekly and were always kept under the safety levels during all the experimentation.

After 21 days, 5 fish per tank were randomly taken and anesthetized in a bath of 30 mg l^{-1} clove oil (Mylonas et al., 2005). Then the fish was left for few additional minutes in the anesthetic, until it ceased ventilating. Then the fish was weight and decapitated swiftly and the brain and liver was extracted. The livers were extracted and weighed in order to calculate the Hepatosomatic Index (GSI) according to the following equation: [(Livers weight (g) / Fish weight (g)) × 100]. Pieces of livers and brains were collect and stored at -80°C for further analyses. Pieces of livers were also fixed in a solution of Formaldehyde:glutaraldehyde (4:1) for histological analysis. For gene expression, aliquots of liver and brains were stored within Rnalater (Ambion Inc., Texas, US) at 4°C until processing.

All procedures involving animals were conducted in accordance the “Guidelines for the treatment of animals in behavioral research and teaching” (“Guidelines for the treatment of animals in behavioural research and teaching,” 2001). The experimental protocol was approved by the Greek National Veterinary Agency with the Protocol Number #255361 to the experimental facility EL91-BIOexp-04.

Histology and area covered with lipid vacuoles in the liver (ACVL%). Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany), sections of livers were dehydrated in gradually increased ethanol solutions (70–96%). Sections of 4 µm were obtained with a microtome (Leica RM2245, Germany) and stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic fuchsine (Polysciences, USA) according to Bennett et al., (1976). Sections were examined under a light compound microscope (Nikon Eclipse 50i) and microphotographed with a digital camera (Jenoptik progress C12 plus).

The estimation of hepatic lipid area was done according to the methodology of Papadakis (Papadakis et al., 2013). For each liver, 6 microphotographs were obtained at ×40 magnification from different areas of the liver. A total number of 300 photographs were analyzed.

Fourier – transform infrared spectroscopy (FT-IR). FT-IR measurements were carried following (Gioacchini et al., 2014). The spectra extracted from the chemical maps were integrated under 2990-2836 cm^{-1} (lipids), 1765-1723 cm^{-1} (COO groups, triglycerides) and 3027-2995 cm^{-1} (=CH groups), where the sum of the integrated areas 2990–2836 cm^{-1} and 1765 – 950 cm^{-1} was indicative of the total cellular biomass of the selected area considered (*cell*). All the values indicative of hepatic fat characteristics were normalized to the *cell* value. These bands were chosen in order to quantitatively locate the presence and the characteristics of fat and were integrated giving a visible display with a false color scale. The intensity of the signal associated with a specific band provides information of the presence and on the localization of the molecular/chemical group.

Measurement of endocannabinoids (AEA, 2-AG) and endocannabinoids-like mediators (OEA, PEA) in liver and brain. The extraction, purification and quantification of EC and EC-like mediators from liver and brain have been performed as previously described (Vincenzo Di Marzo et al., 2001).

Fatty acid amide hydrolase (FAAH) enzymatic activity in liver. The FAAH enzymatic activity was performed according to (Forner-Piquer et al., 2017).

RNA extraction and cDNA synthesis. Total RNA was isolated from liver and brain with RNAzol solution (Sigma Aldrich, Milan, Italy) according to the manufacturer's instructions following (Santangeli et al., 2016). The quantity of RNA isolated was determined using a nanodrop spectrophotometer (NanoPhotometer^{TM}), measuring its purity and quantity at absorbance ratio of 260:280 nm. The quality of the RNA was evaluated using 1% agarose gel electrophoresis.

cDNA synthesis was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500-ng total RNA in a final volume of 100 µl. Reverse transcriptase (RT) reactions were incubated 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without RT.

Real-time quantitative PCR. Real-time quantitative PCR was carried out with an Eppendorf Mastercycler Ep Realplex real-time PCR system (Eppendorf,Wesseling-Berzdorf, Germany), using a 96-well PCR array layout designed for the simultaneously profiling of a panel of 25 regulated genes for liver and 20 for brain (Table 1), selected as markers of: i) endocannabinoid system: *cb1, cb2, pparα, pparβ, pparγ, faah, nape-pld, abdh4, cyt-pla2, cox2, dalgα, abdh6a, abdh12a, trpv1* ii) lipolytic / lipogenic markers*: lepr, lep, fasn, agpat4, dgat1, dgat2, gpat, srebp1, hl, lxr, acox3* iii) appetite: *lepr,, nucb2, nucb1, agrp, pomp, cart, npy*. The array included 24 new sequences for gilthead sea bream, already represented in the IATS-Nutrigroup gilthead sea bream transcriptomic database [(www.nutrigroup-iats.org/sea_breamdb/; (Calduch-Giner et al., 2013)] and uploaded to GenBank with the accession numbers from MG570167 to MG570186. Among them, 21 are full codifying sequences (Table 2). Primers were designed to obtain amplicons of 50-150 bp in length using Beacon designer 7.60. Prior to PCR array analysis, specificity and performance of each pair of primers was assessed by analysis of melting curves and linearity of serial dilutions of RT reactions. Real-time

quantitative PCR was carried out with a Mastercycler Ep Realplex real-time PCR system (Eppendorf. Each PCR-well contained 12.5 µl SYBR Green Master Mix (Bio-Rad), 5 µl of specific primers at a final concentration of 0.9 µM, and 7.5 µl diluted RT reaction. PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The specificity of reactions was verified by analysis of melting curves. Housekeeping gene (β-actin) and controls for general PCR performance were included on each array, and all the pipetting operations were performed with the EpMotion 5070 Liquid Handling Robot (Eppendorf). The efficiencies of all PCR runs were always higher than 90% and specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 over a temperature range of 55–95 °C). Data acquired during the PCR extension phase were normalized using the delta-delta Ct method (Livak and Schmittgen, 2001).

Statistical analysis. Means between the five treatments (in duplicates) of all the examined parameters were analyzed statistically using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Two-ways ANOVA was only used to compare the body weight of the animal before and after the treatments. Statistical significance was set at $p < 0.05$. Results are shown as mean ± SD excepting the HSI and hepatic lipid area which are expressed in percentage and reported as mean ± SD. Data fulfilled the condition for applying a parametric test, given the log-normalization to homogenize the variance when needed and performing ANOVA on ranks were the log-normalization failed. Expression heatmaps of analyzed genes were constructed by means on Genesis software. All statistical procedures were run using *GraphPad Prism 6* and *SigmaStat 3.5.*

Results

BPA and DiNP administered via diet have no effects on body weight and Hepatosomatic index (HSI). The weight of the specimens was measured before and

after the treatment, showing that after 3 weeks of EDC exposure the body weight (Figure 1A) and the HSI were similar to that of control group (Figure 1B).

Alteration of hepatic structure and biochemical composition. Histological analysis of the liver, one of the main target of BPA and DiNP, were performed to determine whether the selected pollutants could induce morphological changes. The histological analysis showed that both EDCs altered the structure of the liver, losing the typical cord conformation and the hexagonal shape of the hepatocytes. Regarding the treated groups, the hepatocytes shifted to a spherical shape due to the accumulation of fat with diverse sizes referable to a heterogeneous steatosis. The morphological changes were more evident among the groups fed with the lowest concentration of both pollutants (BPA LOW and DiNP LOW) while the group DiNP HIGH showed the lowest level of steatosis (Figure 2A). In addition, as it can be observed in the Figure 2B, the area covered by hepatic lipid was increased by all treatments. It was observed that the percentage of tissue covered by lipid was elevated also in the control group probably caused by the high energetic content of the commercial food.

Considering the biochemical composition of the liver assessed by FT-IR (Figure 3), both DiNP and BPA increased the presence of lipids and fatty acids, but it seems that the increase induced by BPA HIGH is higher than in the other groups. Surprisingly, the presence of glycogen was dramatically reduced in all the treatments. In accordance with the FT-IR imaging, the quantitative analysis of lipids and triglycerides (Figure 4) showed an increase in all treatments evidencing the highest levels in the BPA HIGH group. However, the presence of phospholipids was reduced in all the treated groups, being the lowest in BPA LOW treatment. The fatty acid (f.a.) length chain was significantly increased in all groups exposed to the EDCs (Figure 4).

Changes in the EC and EC-like mediator levels after EDC intake. The levels of the endogenous cannabinoids were measured at central level (brain) where AEA, 2-AG

and PEA were drastically reduced in all treated groups except for OEA, which levels remained unchanged with the exception for the DiNP LOW treatment (Figure 5A). At hepatic level, AEA was generally decreased by all treatments as well as PEA and OEA (Figure 5B) while the 2-AG levels were significantly decreased only in the BPA HIGH group. Surprisingly, unaltered levels of the FAAH enzymatic activity were observed among the treatments (Figure 6).

DiNP and BPA induced changes at transcriptional level. Genes coding for ECS pathway components, food intake and lipid metabolism biomarkers were investigated in the brain and liver of sea bream by qPCR array. Regarding brain, relative expression levels are stated in Table 3 and summarized as a heatmap in Figure 7a. A significant down-regulation of *cnr1* was evidenced only at low doses of BPA and DiNP. DiNP LOW treatment also decreased the expression levels of *pparb* in comparison with control, and that of the orexigenic *pro-npy.* In fact, the brain expression of this orexigenic factor was significantly decreased in all four experimental conditions. By contrast, both BPA treatments largely increased the expression of the anorexigenic *cart*. Another anorexigenic biomarker, *pomc*, was also significantly upregulated by the BPA HIGH treatment.

Concerning the liver, relative expression levels stated in Table 4 (heatmap in Figure 7b) evidenced that hepatic expression of *cnr2* is higher than that of *cnr1*. Regarding peroxisome proliferator-activated receptors, *ppara* was by far the most expressed among the three subunits. For the AEA metabolism, the biosynthetic enzymes *nape-pld* and *abdh4* were more expressed than the catabolic *faah*. Instead, regarding the synthetic enzymes involved in 2-AG levels, the expression of mRNA codifying for the catabolic enzymes (*abdh6, abdh12*) are higher respect that for the biosynthetic ones (*dagla*). Among the two isoforms of DGAT, *dgat2* was more expressed than *dgat1*. BPA and DiNP treatments had a large impact on the analyzed hepatic expression, with significant differences in 12 of the 25 selected

genes. Both DiNP treatments and HIGH BPA resulted in a significant downregulation of the hepatic expression of *cnr1*. Expression of *dagla* was decreased by both BPA treatments. Dietary treatments with DiNP significantly decreased the hepatic expression of *fasn* (only LOW DiNP), *abdh6* and *srebp1*. All four dietary treatments significantly decreased *agpat* and *gpat* expression. On the contrary, BPA treatment increased the expression of *pparb*, *pparg* (BPA HIGH), *cyt-pla2* (BPA LOW), *dgat* and *lepa* (BPA LOW). This later gene was also significantly upregulated with the DiNP HIGH treatment.

Discussion

Increasing scientific evidences exist regarding the accidental ingestion of plastic debris by marine species, highlighting their extremely persistence in the environment, their low rate of degradation and the possibility to be introduced along the food chain. As we aforementioned, since plastic additives as DiNP and BPA are not chemically bound to the polymer matrix as well as their extensively uses, they often account for the major leachates from plastics (Lithner et al., 2011). In this regard, the results herein obtained showed that both plastic additives induced alterations in the central and hepatic ECS, as well as at morphological and biochemical level in the liver.

In the brain, ECS plays an orexigenic role favoring food intake via CB1 and AEA/2-AG (Williams and Kirkham, 1999; Colombo et al., 1998; Di Marzo and Matias, 2005; Kirkham et al., 2002). In this study, both BPA and DiNP induced a decrease of AEA and 2-AG levels associate with a downregulation of *cnr1* and *pro-NPY* (orexigenic signal) mRNA in all treated groups. Moreover, based on the expression of genes linked with appetite control, the alteration of the appetite seems more notable in the BPA than in the DiNP groups due to the significant upregulation of *pomc* and *cart*, both coding for anorexigenic signals (Lau and Herzog, 2014) in BPA exposed animals. To gain further insight, the levels of PEA and OEA were measured, both

molecules known as endogenous anorexic molecules acting at peripheral level (intestine) (Hansen, 2014). PEA and OEA exert their activity through PPARα, being PEA significantly less effective than OEA in food intake suppression (Avraham et al., 2013). So, the increase of OEA in *DiNP LOW* could be considered a marker of satiety, resulting the significance of the reduced levels of PEA still not explained.

Thus, BPA and DiNP can negatively regulate the appetite as previously evidenced in *S. aurata* with a decrease of food intake after a BPA treatment (5000 µg/kg/day) (Maradonna et al., 2015). Similarly, in male mice exposed during fetal development to 5 and 5000 µg/kg/day, food intake remained unaltered or decreased (Angle et al., 2013) while in rats (male and female), perinatal exposure to 50 µg/kg/day did not alter the food intake (Wei et al., 2011). Regarding DiNP, its administration at higher dosages in male rats for two weeks and for two years, decreased or did not alter the food intake (Kwack et al., 2010; Lington et al., 1997). Interestingly, a recent *in vitro* study using pico- and nanomolar doses of DiNP reported a decrease of *npy* mRNA expression in human neuronal cells (Rendel et al., 2017), confirming its negative effect on appetite control. Despite a reduction of appetite can be hypothesized at central level also in sea bream, all the treatments induced a higher accumulation of lipids (i.e. fatty acids, triglycerides, ACVL) in liver. This finding suggests an over-activity of the ECS at hepatic level. For instance, in different animal models, it has been demonstrated that the induction of CB1 increases of *de novo* fatty acid synthesis through the transcription factor SREBP1c and its target enzyme, FAS (Osei-Hyiaman et al., 2005) jointly with an elevation of AEA. On the contrary, CB1 antagonists improve lipid metabolism and stimulated β-oxidation (Jourdan et al., 2012). Controversially, even the elevated presence of lipids, our data indicated a reduction of the ECS activity, with a reduced *cnr1*, *srebp1* and AEA suggesting a negative feedback among the ECS signal and the liver. In addition, the gene coding for the enzymes of *de novo* phospholipid synthesis pathway, such as *gpat4* and *gpat* were downregulated. However, *dgat2*, in charge of the last step for the triglycerides biosynthesis (TAG) was upregulated in parallel with the elevated

presence of TAG in the tissue, although the presence of phospholipids was decreased. Phospholipids such as phosphatidic acid (PA) and its derivatives, as the phosphatidylinositol (PI), phosphatidyethanolamine (PE) and phospatidylcholines (PC), are the precursors of the EC (AEA and 2-AG) and EC-like mediators (PEA and OEA) (Astarita et al., 2008; Cadas et al., 1996; Fonseca et al., 2013; Hermansson et al., 2011). Actually, PA is an intermediate step for the synthesis of TAG. Considering these evidences, we could speculate that BPA and DiNP downregulate the lipid biosynthesis, especially until the production of PA, and hence, inducing a downregulation of the endocannabinoids due to the reduction of the substrate, the phospholipids. However, TAGs are still biosynthesized, possibly from fatty acids coming from the mobilization of glycogen as a precursor, which levels resulted significantly downregulated as it can be observed in the FT-IR imaging. Fatty acids, which levels are increased, are probably incorporated in the last step of TAG biosynthesis catalyzed by the enzyme codified by *dgat2* gene*,* which was upregulated in fish exposed to both contaminants, as it has been summarized in the Figure 8. Interestingly, the two genes coding for DGAT, *dgat1 and 2,* the latter presenting a major expression than *dgat1.* According to data available, DGAT2 is more potent than DGAT1 due to its higher affinity for the substrates (Yen et al., 2008).

In addition, a complex regulation of TAG biosynthesis is achieved by SREBP1c, PPARγ/α and LXR's (Coleman and Lee, 2004). SREBP-1c activates genes involved in fatty acid and TAG synthesis, as FAS or GPAT. However, despite the high levels of TAG observed in the livers, *srebp1* was kept downregulated possibly because a negative feedback due to the high levels of TAG, as we previously mentioned. Regarding PPAR's, generally, all the treatments in the present study increased the *ppar*'s expression, being contradictory the upregulation of *ppara* and *ppary* due to their distinct roles. In fact, *ppara* stimulates fatty acid β-oxidation while *ppary* favors lipid biosynthesis and storage, leading to hepatic steatosis. Such upregulation can be explained since xeno-contaminants can interfere with nuclear

transcriptional regulators, as PPAR's, indeed DiNP and BPA are *per se* considered to possess PPAR activity in humans and rodents (Grün and Blumberg, 2009; Kaufmann et al., 2002; Valles et al., 2003). As previously reported (Grün and Blumberg, 2009), PPARs contain some of the largest and most promiscuous ligand binding pockets, allowing PPAR agonists, as EDCs, to activate them.

Regarding other markers of hepatosteatosis, the expression of *lepa* seems not follow the same patter than the receptor (*lepr*), being the ligand upregulated in all the groups. Huang and coworkers (Huang et al., 2006) demonstrated that leptin decreases hepatic TAG levels and TAG secretion, and hence, increases fatty acid oxidation, but our selected markers for fatty acid mobilization including *acox-3* or *hl* were unaffected. Only OEA levels, which mitigates steatosis in liver (Li et al., 2015), was markedly decreased. However, more than an effect in TAG metabolism, human leptin levels have been positively associated with BPA concentration in serum (Rönn et al., 2014) or in perinatal exposure rats to 50 μg BPA/bw/day (Wei et al., 2011) accordingly with the upregulation of *lepa* in the *BPA LOW* group here observed.

Summarizing, herein we demonstrated that in the sea bream the intake of two common plastic compounds altered the cannabinergic signaling, liver composition and the lipid biosynthesis, exerting an obesogenic role probably not through the increase of appetite, but through an altered mobilization of the internal resources as described in Figure 8. Then, the high volume of plastic produced and the inadequate management, may increase the presence of plastic debris and/or plasticizers in the aquatic environments representing a serious threat for wild animals.

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Tables & figures

Table 1. List of selected genes and primers for real-time qPCR.

Table 2. Characteristics of new assembled sequences according to BLAST searches. [a]Gene identity determined through BLAST searches: CB1: cannabinoid receptor 1; CB2: cannabinoid receptor 2; PPARα: Peroxisome proliferator activated α; PPARβ: Peroxisome proliferator activated β; PPARγ: Peroxisome proliferator activated receptor γ; FAAH: fatty-acid amide hydrolase; NAPE-PLD: N-acyl-phosphatidylethanolamine phospholipase D; ABDH4: abhydrolase domain containing 4; CYT-PLA2: cytosolic phospholipase A2; COX2: cyclooxygenase 2; DAGLA: Sn1-specific diacylglycerol lipase alpha; TRPV1: transient receptor potential cation channel subfamily V member I; AGPAT4: 1-acyl-sn-glycerol-3-phosphate acyltransferase delta; DGAT1: diacylglycerol O-acyltransferase 1; DGAT2: diacylglycerol O-acyltransferase 2; LEPR: leptin receptor; LEP: leptin; GPAT: glycerol – 3 – phosphate acyltransferase: ACOX3: Peroxisomal acyl-coenzyme A oxidase 3; NUCB2: Nesfatin-1; NUCB1: nesfatin-1-like peptide; NPY: neuropeptide Y; AGRP: agouti-related protein; POMP: pro-opiomelanocortin; CART: cocaine- and amphetamine – regulated transcript. ACTB: β-actin. [b]Best BLAST-X protein sequence match (lowest E value). [c]Expectation value. [d]Codifying sequence.

Table 3. Relative expression of genes expressed in the brain of gilthead sea bream. Data are the mean ± SEM. All data are referenced to the expression level of *lepr* of control fish with an arbitrarily assigned value of 1. Asterisks (*) indicate significant differences between the treatment and control group ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$). Evaluated by one-way ANOVA with Tukey's post – test ($p < 0.05$).

Table 4. Relative expression of genes expressed in the liver of gilthead sea bream. Data are the mean ± SEM. All data are referenced to the expression level of *trpv1* of control fish with an arbitrarily assigned value of 1. Asterisks (*) indicate significant differences between the treatment and control group ($p < 0.05$); ** ($p < 0.01$); ***

($p < 0.001$); **** ($p < 0.0001$). Evaluated by one-way ANOVA with Tukey's post – test ($p < 0.05$).

Figure 1. Body weight before and after the treatment and hepatosomatic index (HSI). Data are expressed as mean ± standard deviation; letters above each column indicate statistical difference between treated groups and control group ($p < 0.05$, two-ways ANOVA).

Figure 2. (A) Histological slides of livers. Control (a); BPA LOW (b); BPA HIGH (c); DiNP LOW (d); DiNP HIGH (e). Scale bar: 50 µm. (B) Graphic showing the results of the hepatic area covered by lipid vacuoles. Data are expressed in percentage respect the total area of the hepatic tissue as mean± standard deviation. Letters above each column indicate statistical differences among groups ($p < 0.05$, one-way ANOVA).

Figure 3. Chemical maps integrated under the lipids stretching regions (2990-2836 cm^{-1}) for lipids, fatty acid and glycogen for BPA and DiNP. The color represents the total amount of lipid in the tissue. False color scale on the right.

Figure 4. Graphics showing the results from FT-IR imaging. Data are expressed as mean ± standard deviation in a.u. (adimensional units); letters above each column indicate statistical difference between treated groups and control group ($p < 0.05$, one-way ANOVA).

Figure 5. EC and EC-like mediator levels in brain (A) and in liver (B). Levels of AEA measured as pmol / gr tissue; 2-AG, OEA and PEA as pmol / mg tissue. Data are expressed as mean ± standard deviation; letters above each column indicate statistical difference between treated groups and control group ($p < 0.05$, one-way ANOVA).

Figure 6. FAAH enzymatic activity in liver membrane cells measured as pmol / minutes incubation / mg of protein. Data are expressed as mean ± standard

deviation; letters above each column indicate statistical difference between treated groups and control group ($p < 0.05$, one-way ANOVA).

Figure 7. Heat maps of dietary treatments on the gene expression of selected genes in brain (A) and liver (B). Scales of colors represent fold-change values in comparison to control (green, downregulation; red, upregulation)

Figure 8. Summarized findings. Alteration of the synthesis of triacylglycerol and its metabolic pathways. CL, cardiolipin; DAG, diacylglycerol; FA, fatty acid; G-3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol.

Table 1.

Gene name	Symbol	Primer sequence
Cannabinoid receptor 1	*cb1*	GCT CAA CTC CAC CGT CAA CCC AAT C
		CCG CAG GTC CTT GCT CCT CAAC
Cannabinoid receptor 2	*cb2*	TGG TCC TCA CGA AGG CGG AGA AGA
		CCA GCC AGC AAA CAG ATA GAG CCA ATG AC
Peroxisome proliferator activated receptor α	*pparα*	TCT CTT CAG CCC ACC ATC CC
		ATC CCA GCG TGT CGT CTC C
Peroxisome proliferator activated receptor β	*pparβ*	AGG CGA GGG AGG AGT GAG GGA TGA GGG GAG
		CTG TTC TGA AAG CGA GGG TGA CGA TGT TTG
Peroxisome proliferator activated receptor γ	*pparγ*	CGC CGT GGA CCT GTC AGA GC
		GGA ATG GAT GGA GGA GGA GGA GAT GG
Transient receptor potential cation channel subfamily V member I	*trpv1*	GGG TCT ACC TCC TGT ACC TAG TCA TCT TCA C
		GCC AAA CGG ATT CTT TC CAT CCC TCC TAT T
Fatty-acid amide hydrolase	*faah*	CCT CTG GCT CCT CGT ATT GCT TCC
		CAA CCG ATC CCA CTC CAC AGA TAG C
N-acyl-phosphatidylethanolamine phospholipase D	*nape-pld*	CGG CTA CTG CTC TTC CTT CCA
		TGC GAG GTC AAC GGG TCC ATA
Abhydrolase Domain Containing 4	*abdh4*	GTG ACC CTA CCA AAC CAG GAT CGA ATA TG
		CGC TTC GCC TTG TTG GTG ACG
Cytosolic phospholipase A2	*cyt-pla2*	GCT GAT GAA CAG AGT GAG CAG TAA CC
		GCC TGG ATG TAG TTG GTG ATG TGT

Cyclooxigenase-2	*cox2*	GCG GAC GGG ATA TAC TGG ACA A
		GGC GAA GGT TTC AGG GAG ATT T
Sn1-specific diacylglycerol lipase alpha	*daglα*	GCG TCT GGT TGG TGG TTC TG
		AGG ACT GGT CTG AGC CAT ACG
Monoacylglycerol lipase abdh6a	*abdh 6a*	CAT CAC CTC CGT CCT GTT C
		CGC CAG TAC CAG TTG TAG G
Monoacylglycerol lipase abhd12a	*abdh 12a*	CGT GCT GGT GCC TGA CTA C
		CGG CTT CAG TCG GCT CAC
Fatty acid synthase	*fasn*	ACA GGC AGC GTC GGT GCC AGT GGT CTA C
		TCC AGG ACG CAG CCT GCC GCG AAC TAC
1-acyl-sn-glycerol-3-phosphate acyltransferase delta	*agpat4*	CTC GTC ATC TGC TAT GTG TTC
		GCT ACC ATC TGG CTA CTG AT
Diacylglycerol O-Acyltransferase 1	*dgat1*	GGA TGA TGG AGC GCC TCC TAA AG
		CTT TGT AGA ACT CTC TGT CTC CGA ACT G
Diacylglycerol O-Acyltransferase 2	*dgat2*	GTA CAC CGC CTG GCT CAT CTT C
		AGA CGA CCT CCT GCC ACC TT
Hepatic lipase	*hl*	TTG TAG AAG GTG AGG AAA ACT G
		GCT CTC CAT CAG ACC ATC C
Liver X receptor α	*lxrα*	GCA CTT CGC CTC CAG GAC AAG
		CAG TCT TCA CAC AGC CAC ATC AGG
Leptin receptor	*lepr*	GGC GGA ACT GAT TCT ACT CTG
		AGT ATC GGA CCT CGT ATC TCA
Leptin	*lep*	CAG CCT GAT CTC AGA CGA CCT TGA CAA C
		TGA TCC AGG AAT CCA GAC AGC GAA GA
Glycerol-3-phosphate acyltransferase 1	*gpat1*	CGG GGA GTG GCT GCT TAT GGT GAA AGT G
		CTT CAG GAC CCC AAG CTC CTT GAA TGT C
Peroxisomal acyl-coenzyme A Oxidase 3	*acox3*	GGG TGG GCA ACC TCG GTA AG
		TGT GTA GAG CTG GGC GAA GAC
Sterol regulatory element – binding proteins 1	*srebp1*	AGG GCT GAC CAC AAC GTC TCC TCT CC
		GCT GTA CGT GGG ATG TGA TGG TTT GGG
Nesfatin-1	*nucb2*	CGG CTG AAA TCA CAG AAG GTA GAA
		AGG TTG ATG CTC TTG TGC TTG G
Nesfatin-1 like peptide	*nucb1*	CTC GCC TCA GGA TGC TGC TCA AG
		GAG TCT GTA TGT TGG TGC TGT CCA GTT TG
Neuropeptide Y	*npy*	AGG GAT ACC CGG TGA AAC
		ATG TAG TGT CTC AGG GCTG
Agouti-related protein	*agrp*	TGC CTC TTC TTG CTT GCC ATC
		TGT CAT TCT CAG TCT TCC TCA CAT CTT
Pro-opiomelanocortin	*pomc*	TCA CTG CTG AGA CGC CAA TC
		CGG AGG GAG AGGT TGT AGG T
Cocaine- and amphetamine-regulated transcript	*cart*	CTGA GGA GCA AAG AGA TGC CCT TAG AGA AA
		GCG TCA CAC GAA GGC AGC CA
β-actin	actb	TCC TGC GGA ATC CAT GAG A
		GAC GTC GCA CTT CAT GAT GCT

Table 2.

Contigs	Size (nt)	Annotation[a]	Best match[b]	E[c]	CDS[d]	GenBank accession
C2_14935	1491	CB1	XP_010750481	0.0	1 - 1407	MG570167
C2_34590	1233	CB2	XP_019130217	0.0	<1 – 1059	MG570168
C4_03715	2749	FAAH	XP_019135084	0.0	253 - 2013	MG570169
C2_2102	2659	NAPE-PLD	XP_018549114	0.0	<1-1224	MG570170
C4_21189	1255	ABDH4	XP_010745261	0.0	118 - 1221	MG570171
C3_c3781	2479	COX-2	AHC69389	0.0	51- 1874	MG570172
C2_55849	3326	DAGLα	XP_008279582	0.0	1-3222	MG570173
C2_21917	3798	TRPV1	XP_008305127	0.0	159 - 2516	MG570174
C4_00631	1753	AGPAT4	XP_019131188	0.0	229 - 1356	MG570175
C2_40602	1624	DGAT1	XP_019110372	0.0	19 - 1590	MG570176
C2_8832	2168	DGAT2	XP_008285514	0.0	138 - 1220	MG570177
C2_51183	4763	LEPR	AIB06819	8e-147	239 – 3652	MG570178
C2_31952	954	LEP	AHH86062	2e-66	355 – 897	MG570179
C2_30122	3944	GPAT1	XP_018558829	0.0	218 – 2698	MG570180
C2_45643	3121	ACOX3	XP_022067241	0.0	121 - 2196	MG570181
C2_14540	1804	NUCB2	XP_008288466	0.0	74 – 1555	MG570182
C2_8104	1545	NUCB1	XP_019111305	0.0	83 - 1438	MG570183
C2_56854	480	NPY	XP_018523120	1e-42	1-291	MG570184
C2_31112	1051	AGRP	CCF78544	6e-66	75 - 422	MG570185
C3_c27777	611	CART	XP_010747597	4e-68	83 - 1438	MG570167

Table 3.

GENE	CONTROL	BPA LOW	BPA HIGH	DiNP LOW	DiNP HIGH	*p-value*
cb1	7.44 ± 2.59	4.17 ± 1.51*	5.45 ± 1.80	2.16 ± 0.61**	5.45 ± 3.10	0.005
cb2	0.017 ± 0.008	0.021 ±0.004	0.022 ± 0.004	0.025 ± 0.008	0.017 ± 0.007	0.11
ppar α	5.23 ± 2.45	5.34 ± 2.00	3.49 ± 1.48	7.09 ± 2.96	5.56 ± 1.54	0.033
ppar β	1.22 ± 0.26	1.44 ± 0.30	1.26 ± 0.41	1.76 ± 0.39*	1.45 ± 0.39	0.045
ppar γ	1.60 ± 0.29	1.45 ± 0.26	1.40 ± 0.08	1.91 ± 0.27	1.42 ± 0.29	0.002

faah	0.075 ± 0.02	0.121 ± 0.04	0.091 ± 0.05	0.111 ± 0.03	0.105 ± 0.03	0.152
nape-pld	4.31 ± 0.60	4.68 ± 0.65	4.68 ± 1.87	5.60 ± 0.80	4.77 ± 0.89	0.208
abdh4	1.31 ± 0.18	1.67 ± 0.61	1.17 ± 0.33	1.77 ± 0.39	1.40 ± 0.23	0.020
cyt-pla2	0.129 ± 0.05	0.141 ± 0.03	0.139 ± 0.06	0.116 ± 0.03	0.105 ± 0.04	0.328
cox2	0.042 ± 0.02	0.040 ± 0.02	0.061 ± 0.03	0.044 ± 0.02	0.029 ± 0.03	0.089
daglα	7.72 ± 2.57	8.35 ± 1.89	8.83 ± 1.56	8.32 ± 3.22	7.87 ± 1.83	0.873
abdh6a	2.36 ± 0.40	2.31 ± 0.27	2.88 ± 1.00	2.68 ± 0.97	2.25 ± 0.44	0.250
abdh12a	0.81 ± 0.27	1.16 ± 0.48	1.02 ± 0.34	1.01 ± 0.19	0.88 ± 0.17	0.204
nucb2	3.73 ± 1.07	4.02 ± 0.67	3.71 ± 0.54	4.51 ± 1.25	4.28 ± 0.76	0.293
nucb1	6.97 ± 1.21	7.37 ± 1.07	6.61 ± 1.27	7.95 ± 0.78	7.00 ± 0.66	0.106
npy	22.41 ± 9.27	11.40 ± 3.44**	10.32 ± 5.32**	7.99 ± 3.81***	10.01 ± 3.34**	0.0006
lepr	1.06 ± 0.23	1.38 ± 0.34	1.37 ± 0.34	1.12 ± 0.12	1.09 ± 0.23	0.028
agrp	2.37 ± 1.12	2.37 ± 0.88	3.37 ± 1.08	1.57 ± 0.58	1.86 ± 0.87	0.008
pomc	0.060 ± 0.04	0.068 ± 0.03	0.58 ± 0.17*****	0.045 ± 0.03	0.037 ± 0.02	< 0.0001
cart	0.815 ± 0.29	4.75 ± 1.71****	3.11 ± 1.25**	0.317 ± 0.30	0.027 ± 0.02	< 0.0001

Table 4.

GENE	CONTROL	BPA LOW	BPA HIGH	DiNP LOW	DiNP HIGH	*p-value*
cb1	0.31 ± 0.11	0.31 ± 0.07	0.13 ± 0.06**	0.16 ± 0.08**	0.11 ± 0.01**	0.0008
cb2	0.56 ± 0.26	0.42 ± 0.14	0.54 ± 0.24	0.31 ± 0.16	0.43 ± 0.29	0.247
trpv1	1.03 ± 0.14	0.96 ± 0.27	1.24 ± 0.28	1.01 ± 0.29	1.08 ± 0.32	0.429
ppar α	135.1 ± 26.23	144.3 ± 40.89	157.6 ± 24.49	165.1 ± 54.09	176.4 ± 37.42	0.447
ppar β	32.59 ± 12.19	63.61 ± 4.60***	60.81 ± 13.59***	36.28 ± 10.63	35.73 ± 9.84	< 0.0001

ppar γ	28.70 ± 7.19	31.67 ± 4.94	53.91 ± 8.93***	33.44 ± 8.64	37.67 ± 12.57	0.0008
faah	0.80 ± 0.26	0.93 ± 0.15	0.91 ± 0.28	0.74 ± 0.23	0.93 ± 0.34	0.545
nape-pld	68.00 ± 15.93	51.29 ± 11.88	58.83 ± 10.91	59.67 ± 17.80	61.34 ± 16.50	0.448
abdh4	16.66 ± 5.24	21.05 ± 7.11	23.15 ± 8.76	18.41 ± 7.67	19.15 ± 5.96	0.494
cyt-pla2	1.19 ± 0.23	1.71 ± 0.30*	1.58 ± 0.33	1.50 ± 0.35	1.52 ± 0.41	0.085
cox2	0.78 ± 0.19	0.98 ± 0.12	0.57 ± 0.08	0.62 ± 0.21	1.00 ± 0.31	0.002
daglα	1.08 ± 0.19	0.76 ± 0.21*	0.77 ± 0.17*	0.85 ± 0.15	0.99 ± 0.16	0.014
abdh 6a	56.68 ± 19.74	40.87 ± 12.73	40.37 ± 7.85	37.44 ± 7.04*	37.16 ± 10.23*	0.028
abdh 12a	128.9 ± 13.45	163.4 ± 35.37	165.6 ± 34.08	141.1 ± 25.55	128.9 ± 28.60	0.043
fasn	40.47 ± 8.02	44.36 ± 5.60	34.60 ± 11.62	18.75 ± 5.75**	41.17 ± 12.73	0.004
agpat4	0.60 ± 0.13	0.43 ± 0.13*	0.40 ± 0.13*	0.43 ± 0.11*	0.34 ± 0.06**	0.006
dgat1	4.69 ± 1.80	4.33 ± 1.35	4.53 ± 1.22	6.13 ± 1.79	3.56 ± 0.92	0.033
dgat2	140.4 ± 15.43	195.5 ± 26.09*	195.4 ± 27.68*	165.6 ± 28.20	168.3 ± 29.84	0.015
gpat	24.89 ± 5.09	15.80 ± 3.06**	18.18 ± 2.68*	16.23 ± 4.18**	18.88 ± 4.55*	0.003
hl	528.7 ± 103.22	552.0 ± 88.04	638.8 ± 106.20	409.7 ± 108.98	499.0 ± 85.80	0.003
lxrα	77.96 ± 14.86	91.50 ± 12.75	101.90 ± 18.00	68.83 ± 16.15	85.48 ± 16.71	0.005
lepr	31.53 ± 8.43	31.73 ± 6.38	34.28 ± 8.13	28.08 ± 6.96	30.23 ± 6.75	0.618
lep	4.74 ± 1.56	15.87 ± 3.81****	8.36 ± 2.13	6.13 ± 2.95	12.64 ± 4.97**	< 0.0001
acox3	92.46 ± 14.87	98.00 ± 22.59	108.60 ± 21.31	95.78 ± 19.56	89.95 ± 12.92	0.394
srebp1	89.00 ± 18.47	63.77 ± 15.96*	62.30 ± 19.30*	49.87 ± 17.37***	59.30 ± 12.39*	0.003

Figure 1.

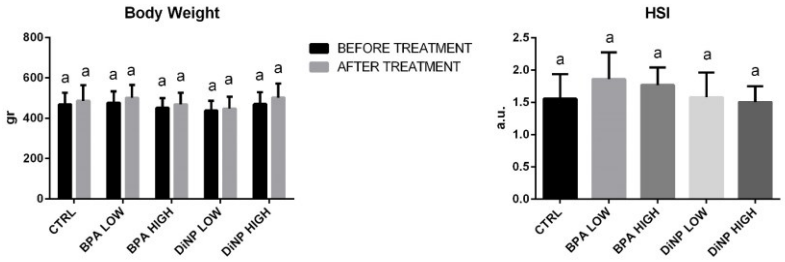
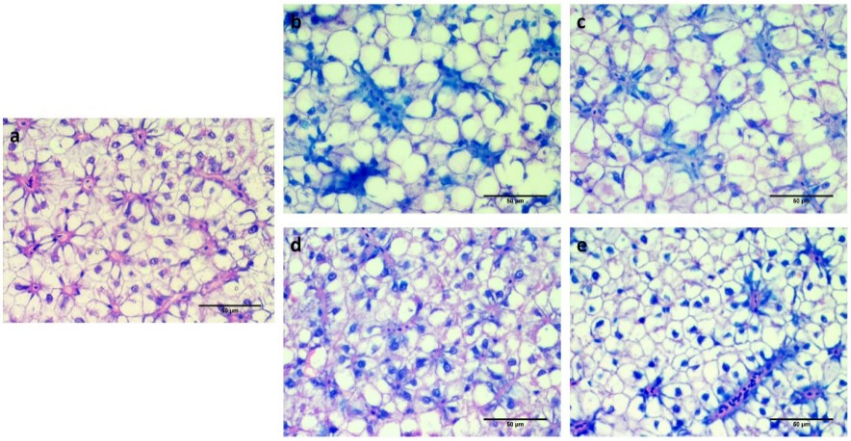


Figure 2A



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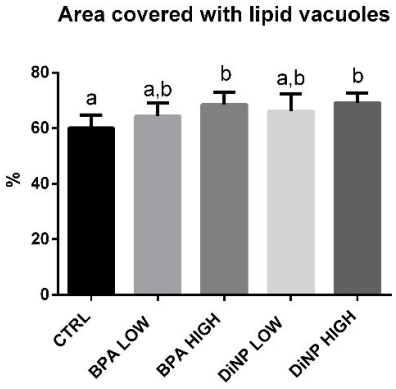

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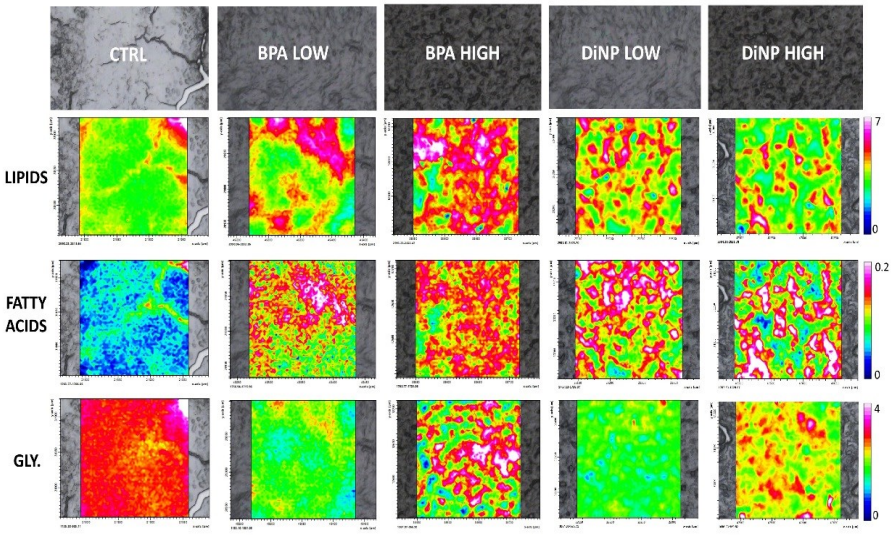

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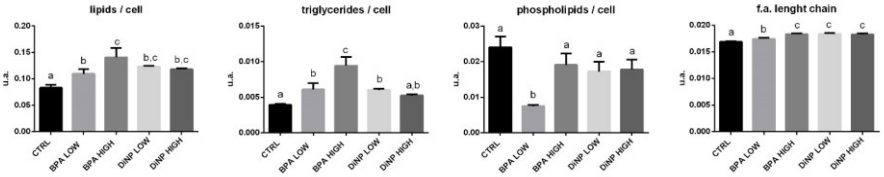


Figure 5A

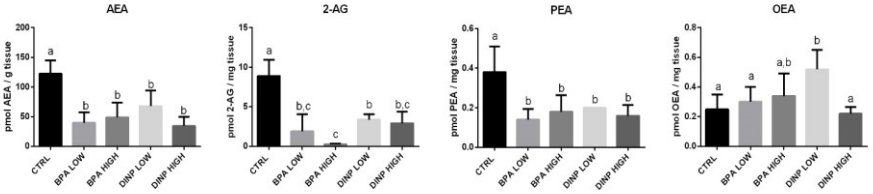
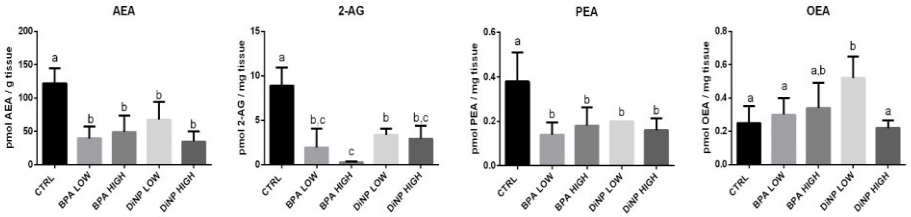


Figure 5B



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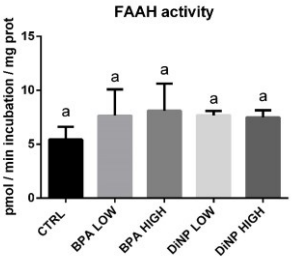
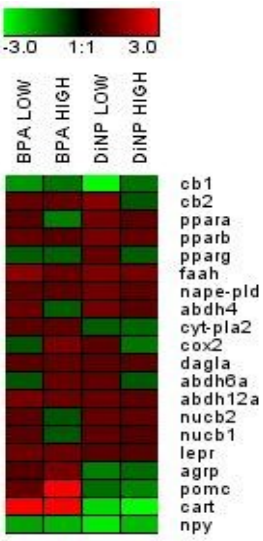


Figure 7

A)



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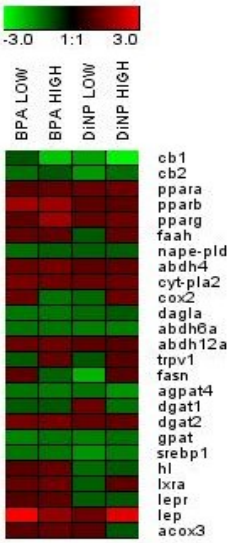

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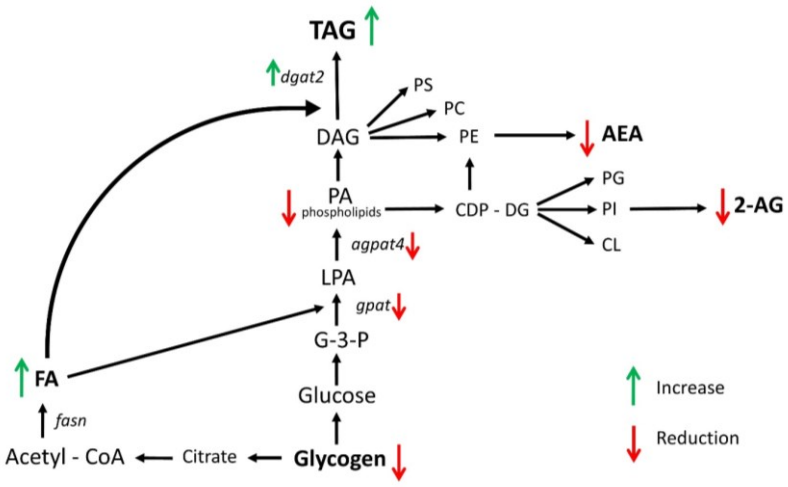
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PS
PC
DAG
PE
AEA
PA
phospholipids
CDP - DG
PG
PI
CL
2-AG
agpat4
LPA
gpat
G-3-P
FA
Glucose
fasn
Acetyl - CoA
Citrate
Glycogen
Increase
Reduction

CHAPTER 5

Effects of the Di-isononyl phthalate on the gonads and plasma steroids of gilthead sea bream

Effects of Diisononyl phthalate via diet on the gonadal endocannabinoid system of adult male S*parus aurata* during the spawning season

Forner-Piquer *et al*.

- *Manuscript in preparation* –

Abbreviated Title: Effects of DiNP in *Sparus aurata*.

Key words: DiNP, endocannabinoids, gonads, gilthead sea bream.

Conflicts of interests: none.

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Abstract

Diisononyl phthalate (DiNP) is a plasticizer added to improve plastic performance and is found in a large variety of items. This compound has been reported by the European Chemical Agency (ECHA) as an anti-androgenic chemical. The endocannabinoid system (ECS) is a cellular signaling system, whose functionality is tightly involved with the reproductive outcome. Thus, the main aim of the present study was the assessment of the effects of the DiNP on the gonadal ECS and reproduction of two-year-old gilthead seabream (*Sparus aurata*), a protandrous hermaphrodite during their first reproductive season as males. Fish were fed for twenty-one days with one of two doses of DiNP, selected based on the Tolerable

Daily Intake (TDI) ruled by the European Commission for humans (*DiNP LOW:* 15 µg DiNP kg^{-1} bw^{-1} day^{-1}*, DiNP HIGH:* 1500 µg DiNP kg^{-1} bw^{-1} day^{-1}). At the end of the treatment period, sperm quality, gonadal histology, gene expression, fatty acid amide hydrolase (FAAH) enzymatic activity, plasma concentrations of steroids and the levels of endocannabinoids and endocannabinoid–like mediators in gonads were determined. The transcription of several genes related to the canabinergic pathway were affected by DiNP, but the effects were not dose-depended. DiNP reduced the levels of endocannabinoids and endocannabinoid–like mediators, concomitantly with an increase of FAAH activity. At the histological level, the low dose of DiNP resulted in a higher frequency of individuals with immature testes. Both DiNP doses showed elevated 17β-estradiol (E_2) levels in the plasma. The results suggest that DiNP may interfere with the endocrine system altering the gonadal ECS, as well as the reproductive function of male gilthead seabream.

Introduction

Phthalates are a group of man-made chemicals used commonly as plasticizers, due to the excellent properties conferred to plastic. Phthalates represented 70% world consumption of plasticizers in 2014 (IHS-Markit, 2015), primarily used in the production of polyvinyl chloride (PVC) products (EPA, 2012). Phthalate- containing products included a large variety of commercial products, however, despite their societal and economic benefits, unfortunately phthalates are not only found in the plastic items we use, but have been identified in the soil, atmosphere and aquatic environment worldwide (Bergé et al., 2013). Additionally, they can be released easily from the products through leaching, evaporation, migration, or abrasion (Bekö et al., 2013). This is because phthalates are not chemically bonded to the resin chains, therefore, they are gradually released from the resin body into the environment (Clausen et al., 2004; Fujii et al., 2003).

Among the family of phthalates, the Diisononyl phthalate (DiNP; CAS No. 28553-12-0 and 68515-48-0) is a high molecular weight ortophthalate used mainly in PVC. As the other members of the phthalate family, DiNP represents an environmental problem due to leaching from a number of products (Roh et al., 2013). Nonetheless, DINP degrades rapidly through abiotic and biological pathways and has a short half-life in water (Lertsirisopon et al., 2009), thus it is not expected to persist in the environment (Staples et al., 1997). Empirical and modelled data indicated that DINP is bioavailable for organisms (Blair et al., 2009; Mackintosh et al., 2004; Mcconnell, 2004), but is not likely to be bioaccumulated or biomagnified in the aquatic food web (Blair et al., 2009; Mackintosh et al., 2004). DiNP has been reported as anti-androgenic chemical (European Chemicals Agency, 2012) in *in vivo* experiments at high dosages. Recent publications showed the potential to disrupt the reproductive processes in zebrafish *Danio rerio* female (Santangeli et al., 2017) and to induce alterations in the endocannabinoid system (ECS) of adult zebrafish (Forner-Piquer et al., 2017) at environmentally relevant concentrations.

The ECS is a lipid-based signaling system involved in the regulation of a plethora of physiological functions, such as reproductive events of mammalian and no-mammalian species (see review by Maccarrone, 2009; Meccariello et al., 2014). Classically, the ECS is formed by the endocannabinoids: N-arachidonoylethanolamide (Anandamide or AEA) and the 2-arachidonoyglycerol (2-AG) and the endocannabinoid-like mediators (or AEA-related compounds) as: N-oleolethanolamine (OEA) and N-palmitoylethanolamine (PEA). The G protein-coupled receptors such as the cannabinoid receptor 1 (CB1) and 2 (CB2). In addition to them, we found the enzymes regulating the endocannabinoid and endocannabinoid-like mediator levels. For instance, for the AEA and AEA-related, the enzyme N-acyl-phosphatidylethanolamine (NAPE-PLD) and abhydrolase domain containing 4 (ABDH4) are in charge of their biosynthesis, while the fatty amide hydrolase (FAAH) and the cyclooxygenase 2 (COX-2) for their deactivation. In the

case of 2-AG, its biosynthesis is regulated by Diacylglycerol Lipase (DAGL) and inactivated by Monoacylglycerol lipase (MGLL).

In the present study, we used gilthead seabream (*Sparus aurata*) as experimental model. The gilthead seabream is a protandrous hermaphrodite teleost fish, which undergoes sex reversion during third year of life (Mylonas et al., 2011). All fish reproduce first as males when they are two years old, and during the third year of life, as many as 80% of the individuals turn into female (Zohar et al., 1978). In addition, gilthead seabream has been farmed intensively with an annual production close to 160.000 tons (FAO, 2015), and it is currently the most important marine farmed fish in the Mediterranean Sea, representing 49% of the total marine species farmed (FEAP, 2015). Hence, the economic importance of this species together with its interesting hermaphroditic reproductive strategy make it a stimulating ecotoxicological model to test the effects of chemicals with endocrine disruptive properties, capable of interfering with the reproductive process. Thus, the objectives of the present study were to examine the effects of DiNP on the reproductive function of male gilthead seabream during their first reproductive season and the effects on the gonadal ECS and gametogenesis.

Material and methods

Fish maintenance. Two-year-old gilthead seabream males (mean weight 458.8 ± 56.8 g; Total length 301.7 ± 10.7 cm) were maintained at the AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Centre for Marine Research (HCMR), Iraklion, Crete, Greece. The fish were maintained in 2-m^3 tanks supplied with shallow well-water under ambient photoperiod and stable temperature ranging between 19-20°C. Measurements of dissolved oxygen, pH and NH_3-N and NO_2-N were done weekly during the maintenance period. The fish were fed to apparent satiation twice per day with commercial feed (IRIDA SA, Greece).

Experimental design. The experimental feed containing DiNP was prepared according to Bjerregaard et al. (2007) and Maradonna et al. (2014) using the commercial feed that the fish were used to be eating prior to the experiment. The amount of DiNP added to the feed were adjusted to give the experimental dose according to the feed ration of fish of this size and at the ambient water temperatures. Fish were divided in 3 treatment groups of 10 individuals in duplicates and were stocked in separate 2-m^3 tanks as described earlier and were maintained for 21 days. Fish were fed 0.7% of their body weight (bw), divided to five feedings per day, in order to ensure all the feed administered was consumed. The treatments were as follows: Control (CTRL) that were fed with the commercial feed mixed with the phthalate vehicle (1.4 ml of EtOH kg^{-1} feed); DiNP LOW were fed with commercial feed enriched DiNP, so that fish consuming their daily ration of feed would receive 15 µg DiNP kg^{-1} bw day^{-1}; and DiNP HIGH were fed with commercial feed enriched with DiNP so that fish consuming their daily ration of feed would receive 1500 µg DiNP kg^{-1} bw^{-1}. The doses chosen for the DiNP were based on the Tolerable Daily Intake (TDI) for humans ruled by the European Food Safety Authority, which is about 150 µg kg^{-1} day^{-1}. The experimental doses used in the experiment were one order of magnitude below and above the TDI. The experimentation was done in the same conditions (tanks, temperature and photoperiod) than the previous acclimation of the animals. Water pH (7,52 ± 0.02) and dissolved oxygen levels (89.2 ± 1.8%) were monitored daily during the experimentation. The water NH_3–N and NO_2–N levels were measured weekly and were always kept under the safety levels during all the experimentation.

After 21 days, all fish per tank were anesthetized in a bath of 30 mg l^{-1} clove oil (Mylonas et al., 2005) to assess the spermiation stage, then five individuals randomly taken from each group were sacrificed. Blood was collected from the dorsal vasculature, behind the anal fin, and stored on ice until centrifugation. The separated plasma was aliquoted and store at -80°C until analysis. Sperm was collected after applying gentle abdominal pressure. Then the fish was left for few

additional minutes in the anesthetic, until it ceased ventilating. Then the fish was decapitated swiftly and the gonads and livers were extracted. The gonads were weighed in order to calculate the gonadosomatic index (GSI) according to the following equation: [(Gonads weight (g) / Fish weight (g)) × 100]. Pieces of gonads were collect and stored at -80°C for further analyses. Pieces of gonads were also fixed in a solution of Formaldehyde:glutaraldehyde (4:1) for histological analysis. For gene expression, pieces of liver and gonads (male part) were stored within Rnalater (Ambion Inc., Texas, US) at 4°C until processing.

All procedures involving animals were conducted in accordance the "Guidelines for the treatment of animals in behavioral research and teaching" ("Guidelines for the treatment of animals in behavioural research and teaching," 2001). The experimental protocol was approved by the Greek National Veterinary Agency with the Protocol Number #255361 to the experimental facility EL91-BIOexp-04.

Sperm assessment. Spermiation condition was evaluated before and after of the treatment using a subjective scale (Papadaki et al., 2018) from S0 to S3 (0 = no milt released, 1 = only a drop of milt released after multiple stripping attempts, 2 = milt easily released after the first stripping attempt, 3 = copious amounts of milt flowing with the slightest pressure). The sperm quality parameters (density, sperm motility and sperm survival) were evaluated only after the treatment and the milt stored at 4ºC. Sperm density was assessed as the number of spermatozoa per milliliters of milt (szoa / mL) using an Neubauer haemocytometer after two step dilutions with 1% physiological saline dilution, with a final dilution of 1:.1701. The sperm motility (%) and survival (days) were evaluated 1 day after the milt collection and every 2 days thereafter, until at least 5% of the cells exhibited forward motility in the field of view. Both were assessed on a microscope slide immediately after mixing 1μL of milt with a drop of saltwater (in duplicate).

Histology. Histological analysis of the gonads was performed according Papadaki et al. (2018). Before embedding in methacrylate resin (Technovit 7100®, Heraeus

Kulzer, Germany), the tissues were dehydrated in gradually increasing ethanol solutions (70–96%). Serial sections of 3 µm were obtained with a microtome (Reichert Jung, Biocut 2035, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to Bennett et al., (1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

Enzyme-Linked ImmunoSorbent Assay (ELISA). For the quantification of testosterone (T), 11-Ketotestosterone (11-KT), estradiol (E_2) and 17,20β-dihydroxypren-4-en-3-one (17,20β-P) or Maturating inducing steroid (MIS) in the plasma, already established and well-described enzyme-linked immunoassays (ELISA) were used (Cuisset et al., 1994; Nash et al., 2000; Rodríguez et al., 2000) with some modifications and using reagents from Cayman Chemical Company (Ann Arbor, Michigan, USA). For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) of the samples for 3 min and then, frozen for 10 min at -80°C. Afterwards, the supernatant organic phase was collected in new tubes and evaporated under a stream of nitrogen (Reacti-vap III, Pierce, Germany). Samples were reconstituted in reaction buffer for running in the ELISA.

RNA extraction and cDNA synthesis. Total RNA was isolated from liver and the testes with RNAzol solution (Sigma Aldrich, Milan, Italy) according to the manufacturer's instructions following (Maradonna et al., 2014). The quantity of RNA isolated was determined using a nanodrop spectrophotometer (NanoPhotometer™), measuring its purity and quantity at absorbance ratio of 260:280 nm. The quality of the RNA was evaluated using 1% agarose gel electrophoresis. cDNA synthesis was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500-ng total RNA in a final volume of 100 µl. Reverse transcriptase (RT) reactions

were incubated 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without RT.

qPCR. Real-time quantitative PCR was carried out with an Eppendorf Mastercycler Ep Realplex real-time PCR system (Eppendorf,Wesseling-Berzdorf, Germany), using a 96-well PCR array layout designed for the simultaneously profiling of a panel of 25 regulated genes for gonads and 3 for liver (Table 1), selected as markers of: i) endocannabinoid system: *cb1, cb2, pparα, pparβ, ppary, faah, nape-pld, abdh4, cyt-pla2, cox2, dalgα, abdh6a, abdh12a, trpv1* ii) reproductive markers*: era, erb, pr, ar, lhr, fshr, gnrhr, 17β-hsd, 3β-hsd, lepr, lep*, vtg, zp1, zp3 . The array included 12 new sequences for gilthead sea bream, already represented in the IATS-Nutrigroup gilthead sea bream transcriptomic database [(www.nutrigroup-iats.org/sea breamdb/; (Calduch-Giner et al., 2013)]. Among them, five are full codifying sequences (Table 2). Primers were designed to obtain amplicons of 50-150 bp in length using Beacon designer 7.60. Prior to PCR array analysis, specificity and performance of each pair of primers was assessed by analysis of melting curves and linearity of serial dilutions of RT reactions. Real-time quantitative PCR was carried out with a Mastercycler Ep Realplex real-time PCR system (Eppendorf. Each PCR-well contained 12.5 µl SYBR Green Master Mix (Bio-Rad), 5 µl of specific primers at a final concentration of 0.9 µM, and 7.5 µl diluted RT reaction. PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The specificity of reactions was verified by analysis of melting curves. Housekeeping gene (β-actin) and controls for general PCR performance were included on each array, and all the pipetting operations were performed with the EpMotion 5070 Liquid Handling Robot (Eppendorf). The efficiencies of all PCR runs were always higher than 90% and specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 over a temperature range of 55–95 °C). Data acquired during the PCR extension phase were normalized using the delta-delta Ct method (Livak and Schmittgen, 2001).

Concentration of endocannabinoids (AEA, 2-AG) and endocannabinoids-like mediators (OEA, PEA). The extraction, purification and quantification of EC and EC-like mediators from the testes part were performed as previously described (Di Marzo et al., 2001). Data are reported as means of n= 5.

Fatty acid amide hydrolase (FAAH) enzymatic activity. The FAAH enzymatic activity was performed according to (Forner-Piquer et al., 2017) in the testes part. Data are reported as means of n= 3.

Statistical analysis. Means between the three treatments (in duplicates) of all the examined parameters were analyzed statistically using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Statistical significance was set at $p < 0.05$. Results are presented as mean ± SD. Results of GSI are expressed in percentage and reported as mean ± SD. Data fulfilled the condition for applying a parametric test, given the log-normalization to homogenize the variance when needed and performing ANOVA on ranks were the log-normalization failed. Superscript letters indicate statistical differences among the treatments and superscript asterisks (*) respect the control. All statistical procedures were run using *GraphPad Prism 6* and *SigmaStat 3.5.* Letters indicate statistical differences among the treatments.

Results

The higher concentration of DiNP increased the GSI. Only the higher dosage of DiNP increased the fish GSI respect the control (Figure 1).

Sperm parameters. In order to evaluate whether DiNP affects the sperm quality, the spermiation stage was evaluated before and after the three-week treatment (Figure 2), where the DiNP LOW showed a reduced spermiation index. Regarding the sperm density (Table 3), there were no statistically differences among the groups. Finally, the sperm survival (days) was reduced in the treatments but not

significantly (Table 4A). A detailed sperm survival per groups in reported in Table 5B.

Gonad morphology. All the specimens sampled presented ovotestis conformation. The ovarian part showed oocytes in previtellogenic stage in all the groups (Fig 3a). Regarding the testicular part, the control group (Fig 3a) as well as DiNP HIGH (Fig 3d) group presented mature testis, with all the different stages of the spermatogenesis, however it can be observed the lower presence of spermatogonia in the latter respect to the control group. Regarding DiNP LOW, the 67% of the individuals examined showed immature testes with spermatogonia predominance (Figure 3c).

DiNP altered the EC and EC-like mediator's levels in the testes. AEA, PEA and OEA decreased in the *DiNP HIGH* group, while 2-AG levels decreased after both treatments (Figure 4A). Accordingly, the enzymatic activity of FAAH increased concomitantly with the increase of DiNP (Figure 4B).

DiNP induced sex hormonal alterations. Regarding the E_2, both DiNP treatments incremented the concentration in the plasma. T was increased only by DiNP LOW, while 11-KT the active form of androgen in fish, was significantly decreased by DiNP HIGH. Finally, 17,20β-P or MIS was reduced after the DiNP LOW treatment (Figure 5).

DiNP induced modifications at transcriptomic level. Once evaluated the ability of DiNP to alter the levels of endocannabinoids and sex hormones levels, qpcr-arrays were performed in the male part of the gonad to evaluate whether DiNP induced transcriptional variations. Regarding the testes, relative expression levels are stated in Table 5 and summarized as a heatmap in Figure 6a. DiNP induced alterations in the expression of genes codifying for *cb1, cb2, pparb, pparg, trpv1, faah, abdh4, dagla, abdh12, lepr, lep, pr, ar* and *lhr.* In the liver, relative gene expressions are reported in Table 6 and summarized as a heatmap in Figure 6b, whit an upregulation of *zp1* and *zp3* in the higher dose of DiNP.

Discussion

Many of the chemicals released into the environment have had only a minimum ecotoxicological test (Kime, 1999), and mostly in murine models or zebrafish, and the effect of these chemicals on other potentially exposed organisms is underestimated. Thus, the present study examined the effects of the novel plasticizer DiNP on gilthead seabream, focusing on the ECS, since several studies reported its implications in the reproductive performance of teleost (Cottone et al., 2013, 2008), and the tight relationship existing between sex-steroids and the ECS (Gorzalka and Dang, 2012; Maccarrone et al., 2000; Rossi et al., 2007). As we expected during the first maturity, all the individuals were functionally males and in spermiation during the spawning season. No changes were found excepting the DiNP LOW group which presented the lowest spermiation index and the higher frequency of individuals with post-spawning testes with elevated plasma E_2. Interestingly, during post-spawning and reproductive quiescence, few spermatozoa can be observed and spermatogonia cells are the predominant cell stage (Chaves-Pozo 2005), similar to what was found in the DiNP LOW group. However, in the DiNP HIGH group, together with the highest levels of E_2, reduced presence of spermatogonia was found. This classically considered "female" hormone is now accepted that it may regulate spermatogenesis (O'Donnell et al., 2001) and have an important role in regulating gene expression in the testis (Schulz et al., 2010). Along this line, Chaves-Pozo (2007) reported that elevated plasma E_2 during short time, accelerated the final events of spermatogenesis and inhibited the proliferation of spermatogonia. Thus, it can be hypothesized that DiNP LOW induced a post-spawning stage with a cessation of spermiation and an increase of spermatogonia, resulting in a reduced spermiation stage and low levels of MIS, whose principal role is the stimulation of milt production in males (Scott et al., 2010). On the other hand, the results of the DiNP HIGH group suggested that the higher dose prevent the formation of spermatogonia in agreement with the highest levels of E_2 and the lower levels of 11-kt. 11-kt has been reported to induce the proliferation of

spermatogonia and thus, suggested as one of the factor involved in the initiation of spermatogonial proliferation (Schulz et al., 2010).

In this line, DiNP has been reported as anti-androgenic in murine models (ECHA, 2013) and reduced levels of 11-KT has been also found in fishes from such contaminant-exposed areas (Goodbred et al., 2015), as it can be observed in the highest DiNP dose group. However, higher levels of T were observed in the treated groups. Interestingly, Corton and Lapinskas (2005) reported that short-term exposure to phthalates may lead to decrease in testosterone, while longer exposure may stimulate Leydig cell proliferation resulting in the increase of testosterone, as observed in our study. Associated with the presence of T, Wong and Zohar (2003) reported that in the testicular portion of the gilthead sea bream gonad, *in vitro* T enhanced the expression of *lhr*, as it was observed here.

Following with the altered biomarkers, *the DiNP HIGH treatment* induced the downregulation of androgen receptor (*ar*) and the upregulation of the progesterone receptor (*pr*) in the testis. In the liver, the zona pellucida proteins (*zp1* and *zp3*) were also upregulated, which are reported to be inducible by E2 in gilthead sea bream liver (Modig et al., 2006), overall suggesting an estrogenic-like activity of the DiNP at high dose.

Regarding the expression of leptin (*lep*) and leptin receptor (*lepr*) were deregulated after the treatments, while DiNP LOW downregulated *lepr* and upregulated *lep*; the exposure to DiNP HIGH upregulated *lepr*, but without effect on *lep.* However, the mechanism by which leptin regulates reproduction remains unclear (Tena-Sempere et al., 1999) and we do not have any explanation about the physiological role exerted by these changes. Anyhow, negative correlation has been reported among the levels of leptin and T (Caprio et al., 1999; Tena-Sempere et al., 1999) in rodent testis, as well as at central level among leptin and the levels of AEA and 2-AG (Di Marzo et al., 2001). Thus, in front of the lack of correlation among the expression

of *lepr* and *lepa* and the levels of T or AEA/2-AG further analysis are necessary to clarify the role of this signal in the gilthead sea bream reproduction.

On the contrary, it is well established that there is a relation among sex-steroids and endocannabinoids. For instance, FAAH promoter possess imperfect estrogen response element (EREs) (Waleh et al., 2002) and thus, the FAAH activity and *faah* mRNA expression can be modulated by E_2 (Ciaramella et al., 2016; Grimaldi et al., 2012), in accordance with the increased FAAH activity concomitantly with the levels of E_2 found in plasma, but no with *faah* gene expression. In this regard, FAAH is one of the main hydrolytic enzyme of AEA, which its administration has been negatively correlated with the levels of LH and T (Scorticati et al., 2004; Wenger et al., 2001), as in the *DiNP HIGH* group but not *in the* DiNP LOW.

The cross-talk of sex hormones and ECS involve also the CB1 (*cnr1*). It has been reported that $CB1^{-/-}$ mice shows lower circulating levels of LH, T and E_2 (Cacciola et al., 2013a, 2013b), interestingly, the results here described showed the inverse case, an upregulation of the *cnr1* and *lhr* transcript with a rise of the plasma levels of T and E_2. Anyhow, the elevated levels of *cnr1* or *cnr2* found in the present study does not match with the lower levels of the ligands, such as AEA or 2-AG, probably a deregulation induced by the treatment as previously described for Bisphenol A (Martella et al., 2016) or DiNP (Forner-Piquer et al., 2017) in zebrafish. However, we have to contemplate the possibility of the accumulation of transcripts during meiosis, which will be translated at later stages, a well described process occurring during spermatogenesis (Monesi, 1965).

Very interesting is that $cnr1^{-/-}$ male mice show normal progression of spermatogenesis and are fertile, although they displayed some other abnormalities (Cacciola et al., 2013b). Thus, what is clear from our results is that DiNP induced *cnr1* upregulation in the highest dose, but it did not seem to affect sperm parameters significantly.

As we mentioned above, there is a discordance among FAAH activity and *faah* transcripts, the latter being downregulated in the DiNP LOW group, the same group which show an elevated FAAH activity and unchanged levels of AEA. In this matter, the N-acylethanolamines PEA and OEA, with similar metabolic pathways than AEA, are known to act as "entourage compounds" (Lambert and Di Marzo, 1999), acting as alternative substrates for FAAH inducing the lower levels of PEA and OEA as found in the treated groups. In addition, OEA is involved in inflammatory processes, then, the decrease of OEA and the increase of *trpv1* transcripts could be likewise a sing of an inflammatory process.

Moreover, PEA and OEA activate peroxisome proliferator activated receptors (PPAR's), on this regard, expression of PPARa, PPARb and PPARg has been detected in spermatogonia and spermatocytes of adult zebrafish (Ibabe et al., 2005); however, their role is still unclear (Froment et al., 2006). Interestingly, a cross-talk exists between PPAR and estrogen receptors-signaling pathways through ERE's via PPPAR/RXR (retinoid X receptor) heterodimer (Keller et al., 1995), existing the possibility that such heterodimer binds to several other ERE's. As previously indicated, FAAH contains in its promoter an imperfect ERE and in addition, FAAH/*faah* can be regulated by estrogens, so it is tempting to speculate that the unexpected downregulation of *faah* could be under the coregulation of PPAR/RXR and related with the downregulation of the *ppar*'s. Regarding the role in the testes, Huang (Huang, 2008) suggested that PPARs may be directly involved in germ cell maturation, being the group of *DiNP LOW* the group with the lower mRNA levels of *pparb* and *pparg* and presenting he higher frequency of immature testes. In addition, PPARa and PPARb are also reported to activate the expression of genes involved in fatty acid transport and oxidation in Sertoli cells (Regueira et al., 2014) suggesting the involvement the PPAR in the testis regression.

Regarding 2-AG, the reduced levels found in the treatments did not fit with the transcriptional levels of the biosynthetic enzyme *dagla* and the catabolic *abdh12a*

which were upregulated in the DiNP HIGH group. However, FAAH can also inactivate 2-AG, which enzymatic activity was higher in the treated groups, and possibly, affecting also the final level of 2-AG in testis.

To conclude, the results herein obtained revealed a complexity of the effects exerted by the DiNP on the ECS and steroidogenesis of adult male gilthead sea bream, inducing different responses in the gonad depending on the dose administered. In the light of our results, it seems that DiNP alters spermatogenesis, exhibiting an estrogenic-like and anti-androgenic potential when administered via food to adult male gilthead sea bream. Thus, special attention should be emphasized on the potential toxicity of this new plasticizer that is used as substitutive of other phthalates, since it may interfere with the common gonadal functions.

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Tables and figures.

Table 1. List of selected genes and forward and reverse primers for real-time qPCR.

Table 2. Characteristics of new assembled sequences according to BLAST searches. [a] Gene identity determined through BLAST searches: cb1: cannabinoid receptor 1; cb2: cannabinoid receptor 2; faah: fatty-acid amide hydrolase 1; nape-pld: n-acyl-phosphatidylethanolamine phospholipase d; abdh4: abhydrolase domain containing 4; cox2: cyclooxygenase-2; dagla: sn1- specific diacylglycerol lipase alpha; gpr55: g protein-coupled receptor 55; trpv1: transient receptor potential cation channel subfamily v member ; lepr: leptin receptor; ltp: leptin a; vtga: vitellogenin a, vtgb: vitellogenin b; zp1: zona pellucida – like domain – containing protein 1; zp3: zona pellucida sperm-binding protein 3; era: estrogen receptor alpha; erb: estrogen receptor beta; pr: progesterone receptor; ar: androgen receptor; lhr: luteinizing hormone receptor; fshr: follicle – stimulating hormone receptor; gnrhr: gonadotropin releasing hormone receptor; 17b-hsd: 17β-hydroxysteroid dehydrogenase 14; 3b-hsd: 3β-hydroxysteroid dehydrogenase delta. [b] Best BLAST-X protein sequence match (lowest E value). [c] Expectation value. [d] Codifying sequence.

Table 3. Sperm density as number of spermatozoa / milliliter (szoa/mL). Data expressed as mean ± SD of 10 fishes. Letters above each column indicates significant differences among groups (one-way ANOVA, $p < 0.05$).

Table 4. Percentage of spermatozoa exhibiting forward motility. (A) Average per treatment. Data expressed as mean ± SD of 10 fishes. Letters above each column indicates significant differences among groups (one-way ANOVA, $p < 0.05$). (B) Percentage of individuals exhibiting forward motility (>5% of motile spermatozoa) in the indicated period of time.

Table 5. Relative gene expression in the gonad of gilthead sea bream. Data are the mean ± SD of 6-7 fish. All data are referenced to the expression level of *cyt-pla2* of control fish with an arbitrarily assigned value of 1. Asterisk superscript (*) indicates significant differences between the control and the treatment (one-way ANOVA), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

Table 6. Relative gene expression in the liver of gilthead sea bream. Data are the mean ± SD of 6-7 fish. All data are referenced to the expression level of *trpv1* of control fish with an arbitrarily assigned value of 1. Asterisk superscript (*) indicates significant differences between the control and the treatment (one-way ANOVA), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

Figure 1. Gonadosomatic Index (GSI). Data showed in percentage as mean ± SD of 10 fishes. Letters above each column indicates significant differences among groups (one-way ANOVA, $p < 0.05$).

Figure 2. Spermiation stage before and after the treatments. (S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt easily released after the first stripping attempt, S3 = copious amounts of milt flowing with the slightest pressure). Letters above each column indicate significant differences among groups (one-way ANOVA, $p < 0.05$).

Figure 3. Histological sections from gonads of gilthead sea bream during the first maturation. Bisexual gonad with testicular (T) and ovarian (OV) tissues with previtelognic oocytes, scale bar: 500 µm (a). Testicular tissue from Control group with all types of cells, including spermatozoa (sz), spermatids (sd), spermatocytes (sc), spermatogonia (sg) (b); *DiNP LOW* (d); *DiNP HIGH* (d); scale bar: 100 µm.

Figure 4. (A) ECS levels of the gonads. Data represented as mean ± SD of 5 fishes. Letters above each column indicate significant differences among groups (ANOVA, $p < 0.05$). AEA showed as pmol / g tissue, while 2-AG, PEA and OEA as pmol/ mg

tissue. (B) FAAH enzymatic activity gonads. Data expressed as mean ± SD of 3 fishes as pmol / minutes incubation / mg protein. Letters above each column indicate significant differences among groups (one- way ANOVA, $p < 0.05$)

Figure 5. Plasma levels of Estradiol, testosterone, 11-KT and MIS. Data represented as mean ± SD of 10 fishes in ng hormone / ml plasma. Letters above each column indicate significant differences among groups (one-way ANOVA, $p < 0.05$).

Figure 6. Heatmaps of dietary treatments on the gene expression of selected genes in testis (A) and liver (B). Scales of colors represent fold-change values in comparison to control (green, downregulation; red, upregulation).

Table 1

Gene name	**Symbol**	**Primer sequence**
Cannabinoid receptor 1	*cb1*	GCT CAA CTC CAC CGT CAA CCC AAT C
		CCG CAG GTC CTT GCT CCT CAAC
Cannabinoid receptor 2	*cb2*	TGG TCC TCA CGA AGG CGG AGA AGA
		CCA GCC AGC AAA CAG ATA GAG CCA ATG AC
Peroxisome proliferator activated receptor α	*pparα*	TCT CTT CAG CCC ACC ATC CC
		ATC CCA GCG TGT CGT CTC C
Peroxisome proliferator activated receptor β	*pparβ*	AGG CGA GGG AGG AGT GAG GGA TGA GGG GAG
		CTG TTC TGA AAG CGA GGG TGA CGA TGT TTG
Peroxisome proliferator activated receptor γ	*pparγ*	CGC CGT GGA CCT GTC AGA GC
		GGA ATG GAT GGA GGA GGA GGA GAT GG
Transient receptor potential subfam. V mem.I	*trpv1*	GGG TCT ACC TCC TGT ACC TAG TCA TCT TCA C
		GCC AAA CGG ATT CTT TC CAT CCC TCC TAT T
Fatty-acid amide hydrolase	*faah*	CCT CTG GCT CCT CGT ATT GCT TCC
		CAA CCG ATC CCA CTC CAC AGA TAG C
N-acyl-phosphatidylethanolamine phospholipase D	*nape-pld*	CGG CTA CTG CTC TTC CTT CCA
		TGC GAG GTC AAC GGG TCC ATA
Abhydrolase Domain Containing 4	*abdh4*	GTG ACC CTA CCA AAC CAG GAT CGA ATA TG
		CGC TTC GCC TTG TTG GTG ACG
Cytosolic phospholipase A2	*cyt-pla2*	GCT GAT GAA CAG AGT GAG CAG TAA CC
		GCC TGG ATG TAG TTG GTG ATG TGT

Cyclooxigenase 2	*cox2*	GCG GAC GGG ATA TAC TGG ACA A
		GGC GAA GGT TTC AGG GAG ATT T
Diacylglycerol lipase alpha	*daglα*	GCG TCT GGT TGG TGG TTC TG
		AGG ACT GGT CTG AGC CAT ACG
Monoacylglycerol lipase abdh6a	*abdh 6a*	CAT CAC CTC CGT CCT GTT C
		CGC CAG TAC CAG TTG TAG G
Monoacylglycerol lipase abhd12a	*abdh 12a*	CGT GCT GGT GCC TGA CTA C
		CGG CTT CAG TCG GCT CAC
Leptin receptor	*lepr*	GGC GGA ACT GAT TCT ACT CTG
		AGT ATC GGA CCT CGT ATC TCA
Leptin	*lep*	CAG CCT GAT CTC AGA CGA CCT TGA CAA C
		TGA TCC AGG AAT CCA GAC AGC GAA GA
Estrogen receptor alpha	*era*	ACT CAT CTT CGC ACA GGA CCT CAT ACT
		GCC TTC AAC ACA GTC GCC CTC A
Estrogen receptor beta	*erb*	GAG GCG AGT GAC CCG TCT GT
		CAC GCT TGG TCC GCT AGT TCT G
Progesterone receptor	*pr*	CAG CTT CCG AAG GTC CTG GC
		GTG TGG AAC AAA AGC GGC CTC A
Androgen receptor	*ar*	GCT GAG GTC GCC ATA TCC CAA CT
		GCC ACT CGC TGA CTT CAC TCT TCA
Luteinizing hormone receptor	*lhr*	GCA GGA CCG AGA TCC GTG ACA
		GGG ACC TCT TTC AGT GGC AGA TGA
Follicle – stimulating hormone receptor	*fshr*	CAC CCC GAC CCA AGA CGA AT
		TAG TGG GAC GGC GGA CAT GA
Gonadotropin releasing hormone receptor	*gnrhr*	CGT CTG ACC TCG ACA ACA ACA CCG TTA T
		CAA AGA GTT GGC GGC ACA CG
17b hydroxysteroid dehydrogenase 14	*17b-hsd*	CTG TCA CGG TGG AGC ATT AC
		GTG GAT TTA TGA GGC GGG TG
3b hydroxysteroid dehydrogenase delta	*3b-hsd*	ACC GTC TTC CAC ATC GCA TCC AT
		TTG AAT ACA CGC CTC CAG AAG CAG C
Vitellogenin a	*vtga*	CTA AGG GGA GCC ACA GCA TAC AAC TAC A
		CTG CCT CAA GGA TCA CAA TAC CAC TAG C
Zona pellucida like domain containing protein 1	*zp1*	AAT TAC ACC AAG CCC CTG AC
		CGA CCA CCT GAA CGT AGA TG
Zona pellucida sperm-binding protein 3	*zp3*	TAG GCT CAC AGG CTC AG ACT CTA AGT TCA T
		CCA ACT GGA ACC GCA GCA CGT TAT CT

Table 2

Contigs	Size (nt)	Annotation[a]	Best match[b]	E[c]	CDS[d]
C2_38345	1243	VTGA	CDK37743.1	0.0	<5 – 1243<
C3_c35382	407	Zp1	XP_011606817.1	5e-67	<61 – 402<
C2_27328	1802	Zp3	XP_010755390.1	0.0	273 - 1547
C2_48308	3547	ERα	CAB51479.1	0.0	1 - 1743
C2_34900	2287	ERβ	AAL82742.1	0.0	414 - 2090
C2_73394	487	PR	AFV61589.1	3e-51	<3 - 293
C2_13475	4819	AR	AEO13404.1	0.0	90 - 2309
C2_15245	2487	LHR	ABI93202.1	0.0	111 - 2183
AY587262	3407	FSHR	AAT01413.1	0.0	191 - 202
C2_11982	1281	GNRHR	AAV71128.1	0.0	1 - 1278
C2_4902	3162	17β-HSD	XP_019129086.1	3e-154	1893 - 2684
C2_784	2234	3β-HSD	XP_010732334.2	0.0	189 - 1310

Table 3

	CONTROL	DiNP LOW	DiNP HIGH	*p-value*
Sperm density (Szoa / mL)	12.2 ± 2.57 x $10^{9\,a}$	14.5 ± 2.29 x $10^{9\,a}$	12.2 ± 4.03 x $10^{9\,a}$	0.18

Table 4A

	CONTROL	DiNP LOW	DiNP HIGH	*p-value*
Sperm survival (days)	8.0 ± 4.0[a]	7.8 ± 3.4[a]	5.2 ± 5,0[a]	0.27

Table 4B

Sperm Survival (days)	CONTROL	DiNP LOW	DiNP HIGH
0 - 5	40%	50%	60%
6 - 10	30%	30%	10%
11- 15	30%	20%	30%

Table 5

GENE	CONTROL	DiNP LOW	DiNP HIGH	*p-value*
cnr1	0.38 ± 0.19	0.39 ± 0.16	0.77 ± 0.27**	0.003
cnr2	0.35 ± 0.11	0.31 ± 0.12	0.55 ± 0.14*	0.007
ppar α	3.33 ± 1.07	2.43 ± 0.75	3.48 ± 1.14	0.133
ppar β	6.41 ± 2.00	3.09 ± 1.31**	6.27 ± 1.88	0.004
ppar γ	12.68 ± 5.08	6.03 ± 2.40*	12.91 ± 4.37	0.025
Trpv1	0.24 ± 0.08	0.42 ± 0.11**	0.37 ± 0.11*	0.007
faah	4.87 ± 1.56	2.72 ± 1.07*	4.49 ± 1.59	0.046
nape-pld	22.71 ± 4.91	17.94 ± 5.03	19.53 ± 6.69	0.215
abdh4	3.69 ± 1.10	2.52 ± 0.72*	3.36 ± 0.74	0.046
cyt-pla2	1.00 ± 0.36	0.97 ± 0.31	1.18 ± 0.26	0.415
cox2	1.43 ± 0.52	1.94 ± 0.46	1.07 ± 0.49	0.018
daglα	4.39 ± 1.37	3.11 ± 1.14	6.49 ± 2.04*	0.001
abdh 6a	4.42 ± 1.01	3.41 ± 1.03	3.36 ± 0.94	0.061
abdh 12a	2.56 ± 0.87	2.58 ± 0.97	3.83 ± 1.26*	0.046
lepr	15.88 ± 7.41	5.318 ± 2.90*	27.51 ± 7.46*	0.0002
Lepa	0.30 ± 0.08	0.42 ± 0.12*	0.31 ± 0.02	0.032
Era	3.04 ± 0.9	3.48 ± 0.45	3.13 ± 0.93	0.597
Erb	11.89 ± 2.02	13.55 ± 2.76	14.79 ± 3.65	0.128
Pr	4.36 ± 1.79	4.33 ± 1.04	7.70 ± 3.11**	0.006
Ar	8.59 ± 2.45	7.05 ± 1.45	6.00 ± 0.98*	0.041
lhr	0.56 ± 0.16	1.04 ± 0.26**	1.10 ± 0.30**	0.001
Fshr	0.46 ± 0.15	0.44 ± 0.15	0.53 ± 0.20	0.602
Gnrhr	5.36 ± 2.43	5.17 ± 1.40	3.74 ± 0.93	0.238
17b-hsd	5.20 ± 1.56	4.77 ± .104	4.23 ± 1.52	0.345
3b-hsd	5.91 ± 1.87	5.69 ± 1.96	5.14 ± 1.85	0.709

Table 6

GENE	CONTROL	DiNP LOW	DiNP HIGH	*p-value*
vtga	0.19 ± 0.11	0.14 ± 0.06	0.21 ± 0.07	0.20
Zp1	0.25 ± 0.09	0.12 ± 0.07	0.54 ± 0.17***	< 0.0001
Zp3	29.08 ± 9.95	19.21 ± 7.32	43.59 ± 10.29*	0.001

Figure 1

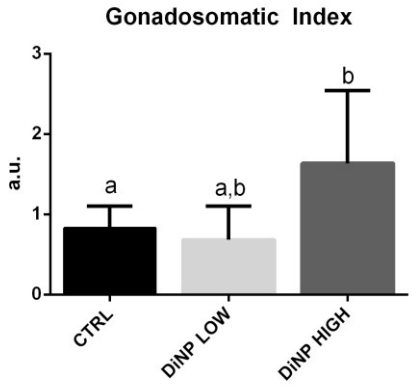


Figure 2

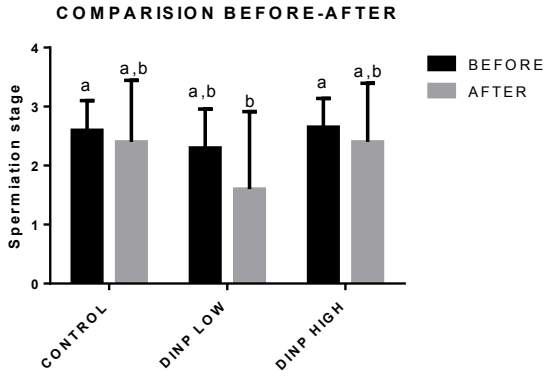


Figure 3

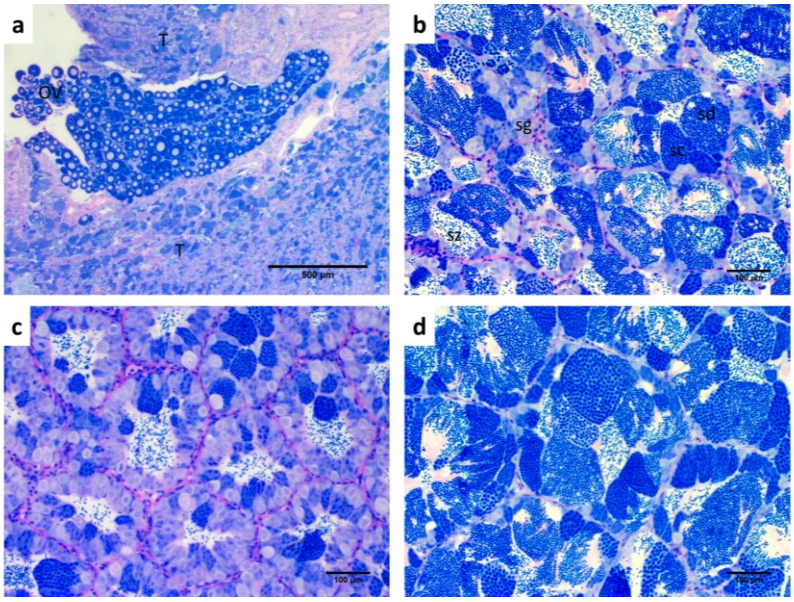


Figure 4A

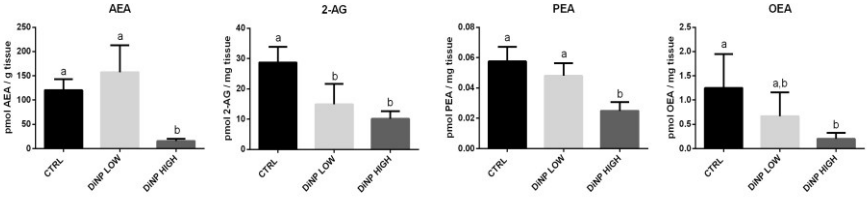


Figure 4B

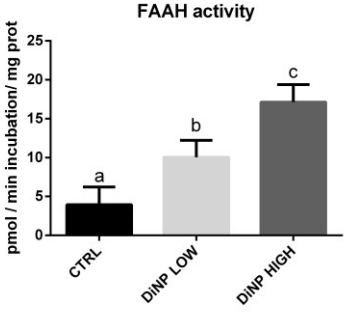


Figure 5

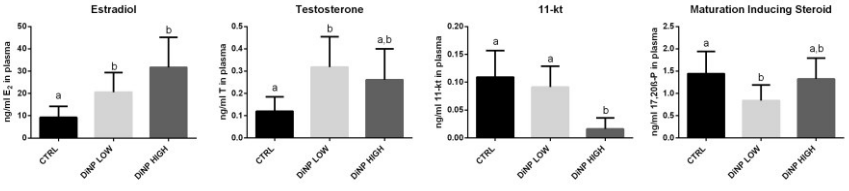
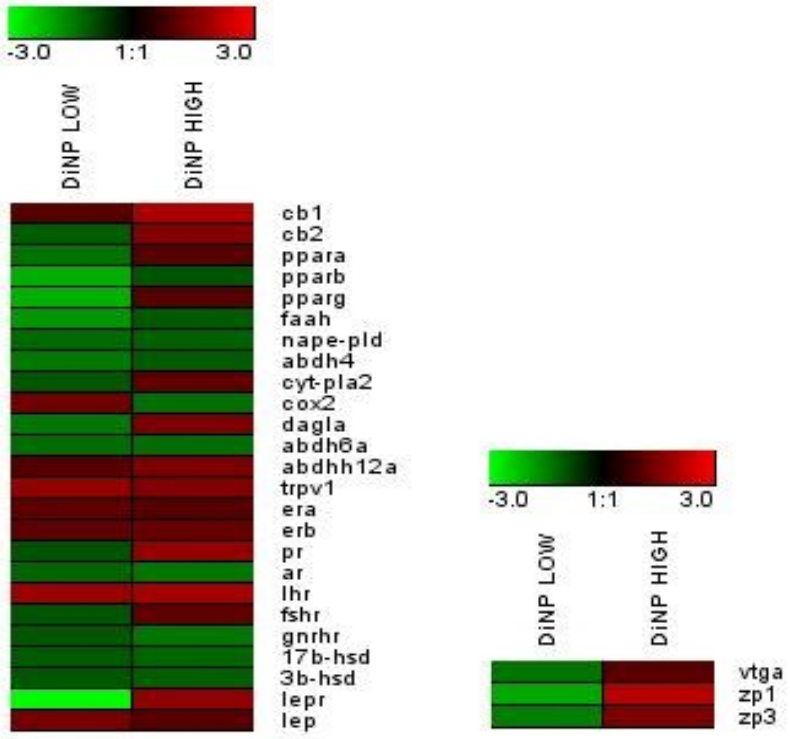


Figure 6

A) B)



GENERAL CONCLUSIONS

The main purpose of present thesis was the evaluation of two plasticizers, ubiquitously found in the environment, on the endocannabinoid system using as experimental model zebrafish (*Danio rerio*) and gilthead sea bream (*Sparus aurata)*. The research performed along this thesis work has approached the remarkable complexity of the endocannabinoid system, a novel system classically studied in humans and murine models.

After a carefully evaluation of the results herein obtained, we can conclude that Bisphenol A and Di-isononyl phthalate induce alterations in the endocannabinoid system signaling at central (brain) and peripheral levels (liver and gonads), modifying the transcriptomic profile of the genes coding for relevant endocannabinoid enzymes as well as the levels of endocannabinoids. Both chemicals induce the disruption of the lipidic pathway at the hepatic level, with the deregulation of the genes coding for enzymes of *de novo* lipid biosynthesis and the alteration of the hepatic biochemical composition with the prevalence of lipids. Both plasticizers alter the expressions of the appetite biomarkers in zebrafish and gilthead sea bream; in addition, the reproductive performance is impaired, being the endocannabinoid pathway deregulated, the fertilization rate decreased, the maturation of the testes affected and the concentration of plasma sex steroids modified.

However, due to the different concentrations and the via of administration of the pollutants (water and food), and hence, their assimilation rate and metabolism, the dose-effect comparation among *Danio rerio* and *Sparus aurata* becomes inappropriate and no accurate, and thus, it has not been performed along the present thesis.

Anyhow, we can confirm that Bisphenol A and Di-isononyl phthalate at environmental range and at the Tolerable Daily Intake levels for humans, have negative consequences for the well-being of the herein studied aquatic organisms.

In the light of the present results, the impairments herein found become alarming due to the wildly uses of Bisphenol A and Di-isononyl phthalate for the plastic manufacture. Indeed, world population and hence, the manufacture of plastics are expected to rise in the following years, for that, a better plastic management, a most efficient recycling system and an improved sewage plant treatment are urgently needed in order to decrease the elevated volume of litter and unclean water ending up to the aquatic environments.

Furthermore, a new revision of the legislation for the uses of Bisphenol A and Di-isononyl phthalate is necessary. The governmental advisory panels for both chemicals should review the new evidences reported by the scientific community and implement new measures for the uses of both plasticizers in plastic items.

In the view of the increase of plastic production reported by the Association of Plastic Manufacturers (*PlasticsEurope*), new consume habits need to be assumed by the population in order to decrease the daily volume of plastic used. New behaviors such as to avoid the “single-use plastics”, replacement of the plastic items for more ecological options as paper or glass, or the implementation of new legislation regarding food containers are urgently required.

Summarizing, our results confirmed the principal hypothesis of the present thesis, being the ECS disrupted by the EDCs. Moreover, both species are extremely useful as ecotoxicological model organisms and in addition, the endocannabinoid system may be stablished as a novel target for endocrine disruption.

APPENDIX

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Effects of diisononyl phthalate on Danio rerio reproduction*

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abstract

Di-isononyl phthalate (DiNP) is a high molecular weight phthalate commonly used as a plasticizer. It was introduced as a replacement for bis (2-ethylhexyl) phthalate (DEHP) which is used in the production of plasticized polyvinyl chloride (PVC). The purpose of this study was to investigate for the first time the effect of DiNP on female reproductive physiology in Danio rerio. Fish were exposed to five different doses of DiNP plus control (0 mg/L; 0.42 mg/L; 4.2 mg/L; 42 mg/L; 420 mg/L; 4200 mg/L) for a period of 21 days. We evaluated fish fecundity, oocyte growth, autophagic and apoptotic processes, as well as changes in morphological and biochemical composition of oocytes, using, qPCR analysis, histology and Fourier transform infrared imaging. The results demonstrate a non-monotonic dose response to DiNP. Greater differences were observed at the lowest (0.42 mg/L) and higher concentrations (420 mg/L; 4200 mg/L) of DiNP. The findings provide evidence that exposure to DiNP adversely affect oocytes growth and matu-ration, leading to abnormal gonadal development and reproduction in zebrafish.